VOLUME 38 MAY, 1959 NUMBER 5

The Journal of Clinical Investigation

EDITED FOR THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

Contents

Serotonin Metabolism in Liver Disease. Francis J. Borges, Jerome K. Merlis and Samuel P. Bessman 715

Calculation of Intracellular pH from the Distribution of 5,5-Dimethyl-2,4-Oxazolidinedione (DMO). Application to Skeletal Muscle of the Dog. William J. Waddell and Thomas C. Butler 720

Effects of Reduced Glomerular Filtration on Urine Concentration in the Presence of Antidiuretic Hormone. Norman G. Levinsky, Douglas G. Davidson and Robert W. Berliner 730

The Role of Urea in the Urine Concentrating Mechanism. Norman G. Levinsky and Robert W. Berliner 741

Toxicity of Urea and its Role in the Pathogenesis of Uremia. Evelyn Frances Grollman and Arthur Grollman 749

Sialic Acid in Human Semen and in the Male Genital Tract. Leonard Warren 755

Transmission of the Common Cold to Volunteers Under Controlled Conditions. IV. Specific Immunity to the Common Cold. George Gee Jackson and Harry F. Dowling 762

The Relation of Urinary CO_2 Tension to Bicarbonate Excretion. Richard M. Portwood, Donald W. Seldin, Floyd C. Rector, Jr. and Robert Cade 770

A Practical Study of the Air Chamber Model of the Cardiovascular System. Sidney Roston and Leonard Leight 777

The Renal Clearances of Iodide and L-Diiodotyrosine Measured Simultaneously. William E. Lassiter and John B. Stanbury 784

The Effect of Triiodothyronine on the Oxidative Metabolism of Erythrocytes. I. Cellular Studies. Thomas Necheles and Ernest Beutler 788

Pyrimidine Metabolism in Man. I. The Biosynthesis of Orotic Acid. Lloyd H. Smith, Jr. and Faith A. Baker 798

Studies on Enhanced Fibrinolytic Activity in Man. Sol Sherry, Robert I. Lindemeyer, Anthony P. Fletcher and Norma Alkjaersig 810

Studies in Kernicterus. I. The Protein Binding of Bilirubin. Gerard B. Odell 823

Effect of Altered Intrathoracic Pressure on Renal Hemodynamics, Electrolyte Excretion and Water Clearance. H. V. Murdaugh, Jr., H. O. Sieker and F. Manfredi 834

The Effects of Adrenal Steroids and Potassium Depletion on the Elaboration of an Osmotically Concentrated Urine. Gerhard Giebisch and Rodolfo Lozano 843

Studies on a Case of Essential Hyperlipemia. Blood Lipids, with Special Reference to the Composition and Metabolism of the Serum Glycerides Before, During and After the Course of a Viral Hepatitis. Lars A. Carlson and Börje Olhagen 854

Comparative Studies on the Half-Life of I^{131}-Labeled Albumins and Nonradioactive Human Serum Albumin in a Case of Analbuminemia. Hermann Bennhold and Ekkehard Kallee 863

The Journal of Clinical Investigation

Printed by the Lancaster Press, Inc., Prince and Lemon Sts., Lancaster, Pa. Published by the American Society for Clinical Investigation. Publication Office, 333 Cedar Street, New Haven 11, Conn.

PHILIP K. BONDY, EDITOR-IN-CHIEF

ASSOCIATE EDITORS

FRANKLIN H. EPSTEIN ALLAN V. N. GOODYER

The Editorial Committee of the American Society for Clinical Investigation

THOMAS P. ALMY, *New York, N. Y.*
SOLOMON A. BERSON, *New York, N. Y.*
FRANCIS P. CHINARD, *Baltimore, Md.*
GEORGE L. CURRAN, *Kansas City, Kan.*
LEWIS DEXTER, *Boston, Mass.*
I. S. EDELMAN, *San Francisco, Calif.*
LEONARD P. ELIEL, *Oklahoma City, Okla.*
CLEMENT A. FINCH, *Seattle, Wash.*
ALFRED P. FISHMAN, *New York, N. Y.*
ROBERT A. GOOD, *Minneapolis, Minn.*
JOHN B. HICKAM, *Indianapolis, Ind.*
ARNOLD S. RELMAN, *Boston, Mass.*
I. HERBERT SCHEINBERG, *New York, N. Y.*
JOHN V. TAGGART, *New York, N. Y.*
RICHARD W. VILTER, *Cincinnati, O.*

THE JOURNAL OF CLINICAL INVESTIGATION is designed for the publication of original investigations dealing with or bearing on the problems of disease in man, and it is the policy of the editors that the JOURNAL should cover the field of clinical investigations in its broadest sense.

Contributions should be sent to the **Editor-in-Chief, Dr. Philip K. Bondy, 333 Cedar St., New Haven 11, Conn.** In order to hasten the process of reviewing and editing it is requested that each original manuscript be accompanied by a duplicate copy and duplicate illustrations.

In order further to expedite the earliest possible publication of articles submitted to the JOURNAL OF CLINICAL INVESTIGATION they will be published in the order of final processing after review, correcting and editing rather than in the strict sequence of their receipt in this office. Papers which require much editorial change, revision of charts and tables, condensation and rewriting will be delayed according to the time required for their correction. This policy will put a premium on excellence of manuscript as well as the major consideration of content. It is requested that figures be photographed, the photographs accompanying the manuscript. The originals of the figures need not be forwarded except by special request of the editors. For further instructions regarding the preparation of manuscripts, see the first page of the January, 1959, issue.

Each volume will consist of 12 monthly issues appearing throughout the calendar year. The subscription price is $15 per volume, **payable in advance**; foreign countries also $15, payable in U. S. funds. Single copies will be sold when available at Vols. 1–28, $2; Vols. 29–38, $1.50 a copy. Authors may purchase reprints at scheduled prices. **A special subscription rate of $6 per volume of 12 monthly issues is offered to medical students, interns, fellows, and residents of approved hospitals.** Subscriptions are **net postpaid.** Remittance should be made by draft, check or postal money order, payable to **The Journal of Clinical Investigation, 333 Cedar Street, New Haven 11, Conn.** Claims for copies lost in the mails must be received within **thirty** days (domestic), **ninety** days (foreign), of the date of issue. Changes of address must be received at least two weeks in advance of issue.

Second-class postage paid at Lancaster, Pa.

Copyright © 1959, American Society for Clinical Investigation

100

a significant

Sanborn achievement in electrocardiographs

VISO

the completely new, 2-speed

SANBORN Model 100 VISO-CARDIETTE

Here is an electrocardiograph in which *no detail* has been overlooked to give you diagnostically *accurate* information ... the greatest possible operating *convenience* ... and modern, *functional* attractiveness. With thirty-five years of experience, this is the finest electrocardiograph Sanborn Company has ever produced. Priced at eight hundred fifty dollars, delivered continental U. S. A.

SANBORN COMPANY

MEDICAL DIVISION • 175 Wyman St., Waltham 54, Mass.

Book News from **LITTLE, BROWN**

MECHANISMS OF HYPERSENSITIVITY

Henry Ford Hospital International Symposium

Just off press! The whole broad field of immunologic processes as they pertain to problems of hypersensitivity is assessed for the first time in this volume prepared by sixty-five investigators and clinicians from all parts of the world.

A few of the many topics covered are factors that condition the individual to undergo sensitization, a careful consideration of antibodies, new aspects of physiological control of histamine in allergy, and the first comprehensive review of evidence for participation of serum complement in allergic responses.

Since hypersensitivity response plays an increasingly important role in many areas of medicine and surgery this book is especially timely. Dermatologists, allergists, immunologists and others engaged in basic research in this field will find it an indispensable reference.

768 pages 230 illustrations $18.50

Smart

METABOLIC DISTURBANCES IN CLINICAL MEDICINE

An invaluable aid on all disturbances of metabolism neatly organized in one small volume. Chapters include Problems of General Nutrition in Clinical Medicine, Atherosclerosis, Lung Function and Acid-Base Balance, and Disturbances of Metabolism affecting the Central Nervous System.

358 pages illustrated $10.00

Kline

PSYCHOPHARMACOLOGY FRONTIERS

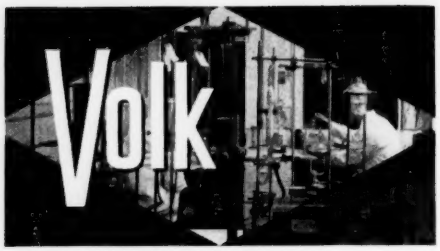
The world's leading psychiatrists, pharmacologists, psychologists, and neurologists present their latest findings on the use of drugs in the treatment of mental illness. This book faithfully records the largest program ever devoted to psychopharmacology.

560 pages 49 illustrations $10.00

at your medical bookstore or from

LITTLE, BROWN AND COMPANY

34 Beacon Street, Boston 6, Mass.



The Singular Source for Isotopic Compounds . . per your exact specifications

Over 550 labeled compounds of guaranteed radiopurity regularly available including:

carbon-14
sulfur-35
phosphorus-32
deuterium
tritium

Available by arrangement:

a. Special packaging and sizes.
b. Special forms; e.g., solutions for injection, crystalline solids.
c. High, low, or standard specific activity.

Unconditional Quality Guarantee.

Write for our new comprehensive 24-page catalog.

Volk Radio-Chemical Company
5411 North Clark Street, Chicago 40, Ill.

WANTED

Back Issues of

THE JOURNAL OF CLINICAL INVESTIGATION

January, July, (Pt. I), September, through December, 1955

January through December, 1956

75¢ per copy and postage

For particulars write to

The Journal of Clinical Investigation
Business Office
333 Cedar Street
New Haven 11, Conn.

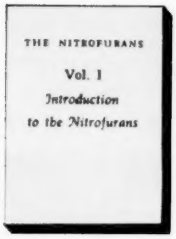
TWO NEW REFERENCE WORKS ON

THE NITROFURANS

a class of wide-spectrum antimicrobials whose efficacy remains undiminished—and whose applications continue to multiply—after 15 years of extensive use.

The first clinical preparation of a nitrofuran derivative was introduced toward the end of World War II, to treat wounds incurred in the Normandy invasion.

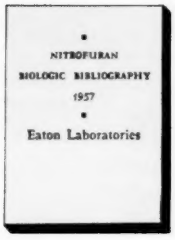
Today, the nitrofurans stand in the forefront of another struggle—the conquest of "resistant staph. infections."



INTRODUCTION TO THE NITROFURANS

traces the development of the nitrofurans from their historical origins to their present status as clinical antimicrobial agents.

Upon request.



NITROFURAN BIOLOGIC BIBLIOGRAPHY

a compilation of all references to the nitrofurans in the medical literature; to be revised annually.

Upon request.

The Nitrofurans . . .
a unique class
of antimicrobials . . .
neither antibiotics
nor sulfonamides

EATON LABORATORIES, NORWICH, NEW YORK

Please send me:

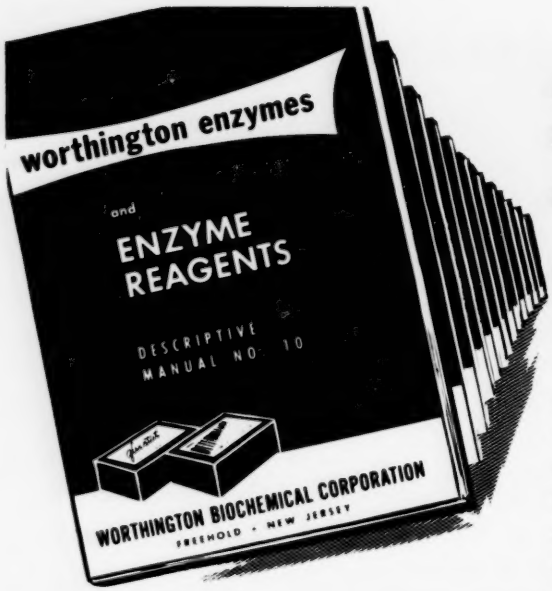
INTRODUCTION TO THE NITROFURANS No. of copies .....

NITROFURAN BIOLOGIC BIBLIOGRAPHY No. of copies .....

Name.....

Address.....

City, Zone, State.....



a synonym for enzymes

worthington

The WORTHINGTON MANUAL #10 is now available.

It is an invaluable handbook on enzymes, inaugurating the use of a new, revised, standardized unitage, as recommended by the Enzyme Section of The Committee on Biological Chemistry of the National Research Council.

For your copy, write:

WORTHINGTON BIOCHEMICAL CORPORATION

FREEHOLD, NEW JERSEY

NOW OVER **5100** **BIOS** CHEMICALS

- p-Carbamylphenylarsine Oxide
- Carbobenzoxy-1-glutamyl-1-tyrosine
- Carboxybenzenesulfonamide
- p-Carboxyphenylarsinoxide
- 5-Carboxyuracil (Uracil-5-carboxylic acid)
- Carnaubic Acid
- Carnaubyl Alcohol
- Caronamide
- Carrageenic Acid
- Carrageenin
- Caryophylline
- Cellotriose
- Cellulase
- Cerium Fluoride
- Cerium Metal
- Cerotic Acid
- Cetyldimethylamine
- Chelidamic Acid
- Chelidonic Acid

Ask for our new complete catalogue

BIOS *Laboratories, Inc.*

17 West 60th St. New York 23 N. Y.

PLaza 7-8171

Because of lack of storage space we must reduce our inventory of the following Supplements

Order while still available

Symposium on Malaria
May, 1948, Part II $2.00

Symposium on Antibiotics
September, 1949, Part I $2.00

Symposium on Radioactive Isotopes
November, 1949, Part I $2.00

Treatment of Acute Infectious Hepatitis
July, 1955, Part II $1.50

The Renal Lesion in Epidemic Hemorrhagic Fever
January, 1957, Part II $1.50

Metabolic Studies in Paralytic Acute Anterior Poliomyelitis
June, 1957, Part II $1.50

* * * * *

Studies on Influenza in the 1957–1958 Pandemic
January, 1959, Part II $1.50

Order through the Business Office of

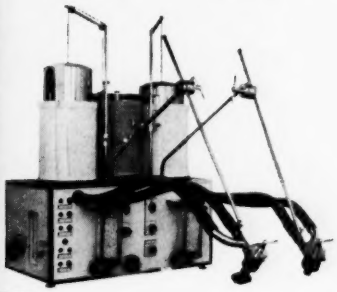
THE JOURNAL OF CLINICAL INVESTIGATION

333 Cedar Street New Haven 11, Conn.

PULMONARY-FUNCTION EQUIPMENT

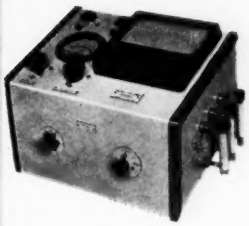
GODART PULMOTEST

The universal double spirometer, the most compact versatile lung function station for determination of lung volumes and its subdivisions. Maximum breathing capacity, timed vital capacity, and oxygen consumption at rest or in exercise can be determined. The GODART PULMOTEST enables the subject to breathe room air, oxygen being added as it is used, maintaining starting concentration, 3-speed kymograph, motor blower, volume integrator, suitable for closed circuit residual volume determination in conjunction with the PULMO-ANALYSOR.



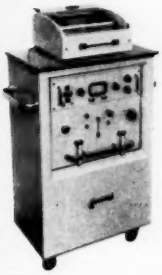
GODART PULMO-ANALYSOR

The PULMO-ANALYSOR is a highly accurate thermal conductivity gas analysor which is sensitive to He, CO_2 and O_2. Usable alone and in conjunction with the GODART PULMOTEST, it can be used to determine residual volume by closed circuit helium method as well as by the open circuit method. Expired gas analysis for O_2 and CO_2 in mixtures with the highest accuracy, speed and precision.



CAPNOGRAPH

This infra-red gas analysor is used for recording dynamic changes in the level of CO_2 in respiration. The response time is rapid (.04 sec.) and is able to follow the respiratory cycle. Alveolar CO_2 values are obtainable without recourse to sampling devices. Capacity is highly stable and accurate to 0.1% of its measuring range, compact and ready for immediate use in proper control of artificial respiration, determination of dead space volume, diagnosis of emphysema, chronic hyperventilation.



GODART COMPLIANCE TEST

To study the elastic properties of the lungs. This equipment records as a kymograph, a loop representing the relation between pressure and volume during respiration. Intra-esophageal pressure changes are detected from a catheter by an electromanometer, volume changes by a precision spirometer and fed into special recorder type kymograph. Supplied with an electromanometer, 3-speed kymograph and all accessories.

GODART DESIGNED PULMONARY FUNCTION APPARATUS

Offices: New York • Utrecht • London

INSTRUMENTATION ASSOCIATES

Distributors of Laboratory and Scientific Specialties

17 West 60th Street New York 23, N. Y.

For Quality without Question... Enjoy the unique refreshment of sparkling Coca-Cola

SIGN OF GOOD TASTE

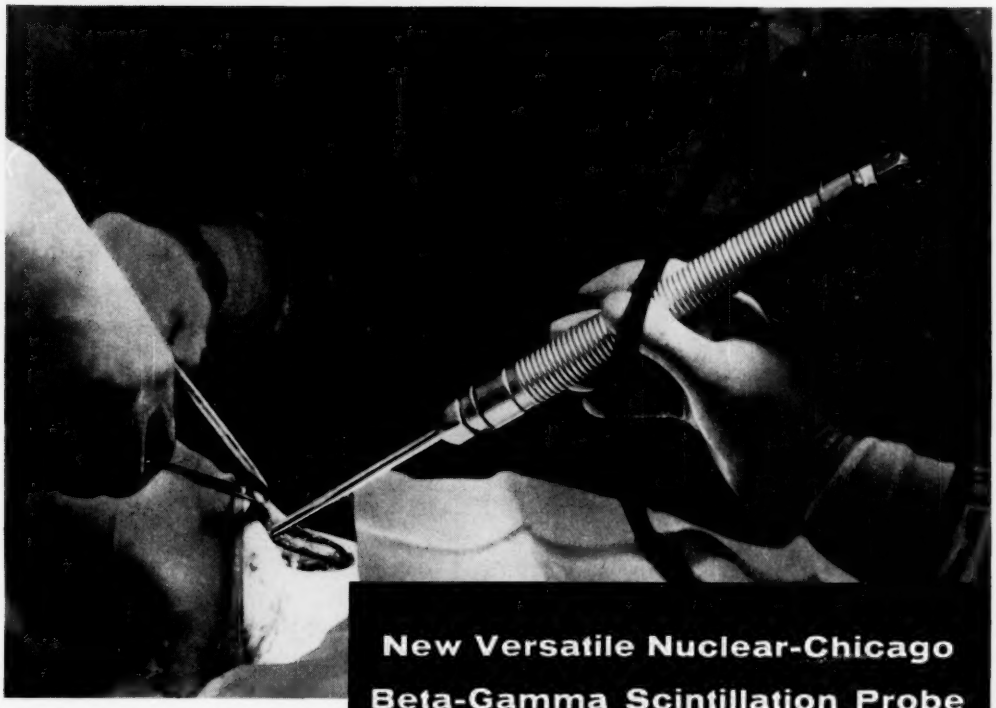
The Journal of Clinical Investigation

333 Cedar Street **New Haven 11, Conn.**

COMPLETE VOLUMES OF BACK ISSUES

Vols. 1 through 11, inc.—special price	$25.00
Vol. 13 (1934) six issues	10.00
Vol. 27 (1948) six issues and one supplement	10.00
Vol. 28 (1949) six issues and two supplements	10.00
Vol. 29 (1950) twelve issues	10.00
Vol. 30 (1951) twelve issues and one supplement	12.00
Vol. 31 (1952) twelve issues	12.00
Vol. 32 (1953) twelve issues	12.00
Vol. 33 (1954) twelve issues	12.00
Vol. 36 (1957) twelve issues and two supplements	15.00
Vol. 37 (1958) twelve issues	15.00
Index, Vols. 1–17, inc.	2.00

Order While Available



Localizing accumulated radioiodine in thyroid remnants with the 6 mm needle probe to assure complete thyroidectomy.

New Versatile Nuclear-Chicago Beta-Gamma Scintillation Probe

Needle probes are provided in these sizes:
2 mm x 10 cm (2.5 mil stainless steel wall)
3 mm x 10 cm (2.5 mil nickel wall)
6 mm x 15 cm (2.5 mil nickel wall)
All probes are easily interchanged by loosening and tightening a simple hand nut.

Designed for the surgeon, radiotherapist or gynecologist, the new Nuclear-Chicago Model DS8 Scintillation Detector is ideal for application in brain tumor localization, pinpointing radioisotope concentrations in thyroid or neoplastic tissue, and estimating radiation doses in radiotherapy. The detector, with three unique, interchangeable needle probes, is a compactly-designed, convenient, lightweight instrument with the complete reliability and accuracy so essential to medical applications.

This rugged detector weighs less than 5 ounces and is only one inch in diameter. It can be sterilized in solutions commonly used in the operating room, is corrosion-proof, and its quality components provide practically infinite life. The beta-gamma sensitive crystals are mounted in the needles in such a way that the active volume is confined to the very tip of the probes.

For more than 12 years we have been designing and manufacturing for the medical profession high quality instrumentation for detecting and measuring radioactivity. We will be pleased to provide you with full information on the Model DS8 Scintillation Probe and on other Nuclear-Chicago instruments for clinical use.

Radioisotopes are creating new frontiers in medicine

nuclear-chicago
CORPORATION
269 WEST ERIE STREET • CHICAGO 10, ILLINOIS

V-CILLIN K®...

dependable, fast, effective therapy

V-Cillin K produces therapeutic blood levels in all patients within five to fifteen minutes after administration—levels higher than those attained with any other oral penicillin. Infections resolve rapidly. *Dosage:* 125 or 250 mg. three times daily. *Supplied:* In scored tablets of 125 and 250 mg. (200,000 and 400,000 units).

New: V-Cillin K® Sulfa. Each tablet combines 125 mg. of V-Cillin K with 0.5 Gm. of the three preferred sulfonamides.

New: V-Cillin K, Pediatric, a taste treat for young patients. In bottles of 40 and 80 cc. Each 5-cc. teaspoonful provides 125 mg. of V-Cillin K.

V-Cillin K® (penicillin V potassium, Lilly)
V-Cillin K® Sulfa (penicillin V potassium with triple sulfas, Lilly)

ELI LILLY AND COMPANY • INDIANAPOLIS 6, INDIANA, U.S.A.

933220

SEROTONIN METABOLISM IN LIVER DISEASE *

By FRANCIS J. BORGES, JEROME K. MERLIS AND SAMUEL P. BESSMAN

(From the Departments of Medicine, Neurophysiology and Pediatrics, University of Maryland, School of Medicine, Baltimore, Md.)

(Submitted for publication August 25, 1958; accepted January 2, 1959)

Serotonin, (5-hydroxytryptamine, 5-HT, enteramine) is widely distributed in animal tissues and has been shown to have marked pharmacologic effects upon the gastrointestinal tract and cardiovascular system (1–4). Indirect evidence suggests that serotonin may play an important role in cerebral function, but, as yet, no direct connection has been established between serotonin and cerebral disease (5). Previous reports (6) and personal observations have shown a low normal excretion of 5-hydroxyindoleacetic acid, the end product of serotonin metabolism, in liver disease. The precursor of the serotonin of brain is apparently 5-hydroxytryptophan. In view of these facts, the possibility that severe liver disease by, in some way, affecting the production of 5-hydroxytryptophan, might alter brain function, was investigated by the following series of experiments.[1]

MATERIALS AND METHODS

Ten to 23 mg. of 5-hydroxy-DL-tryptophan (5-HTP) sterilized by dry heating at 100° for one hour and dissolved in 100 ml. of 0.85 per cent saline solution was given intravenously over a 30 minute period to four patients in severe hepatic coma, to three patients with abnormal electroencephalograms but not in hepatic coma, and to one normal individual. Electroencephalograms were recorded before, during and after infusion. Where possible, 24 hour urine specimens were collected in 6 hour fractions, just prior to, and immediately after, 5-HTP infusion, and analyzed for 5-hydroxyindoleacetic acid (5-HIAA) by the procedure of Udenfriend, Titus and Weissbach (8). Platelet 5-HT content was determined by the butanol extraction procedure of Udenfriend, Weissbach and Clark (9) just prior to and one, two, and three hours after 5-HTP infusion. Blood ammonia levels were determined by the method of Seligson and Seligson (10) as modified in this laboratory.[2]

* Supported by grants from the Heart Association of Maryland and from the National Institutes of Health (B-1365), United States Public Health Service.

[1] A preliminary report (7) of this work has appeared.

[2] Mimeographed copies of the modification will be supplied upon request to Dr. Bessman.

RESULTS

The electroencephalograms of all patients with hepatic failure exhibited the usual diffuse slow activity. During infusion of 5-HTP, all four patients (Table I) showed a diminution of the slow activity and an increase of fast activity, representing a change in the record toward a more normal pattern. However, none of the patients developed a completely normal pattern. Figure 1 shows samples of tracings from Patient M. B. before and during infusion. These changes lasted for two

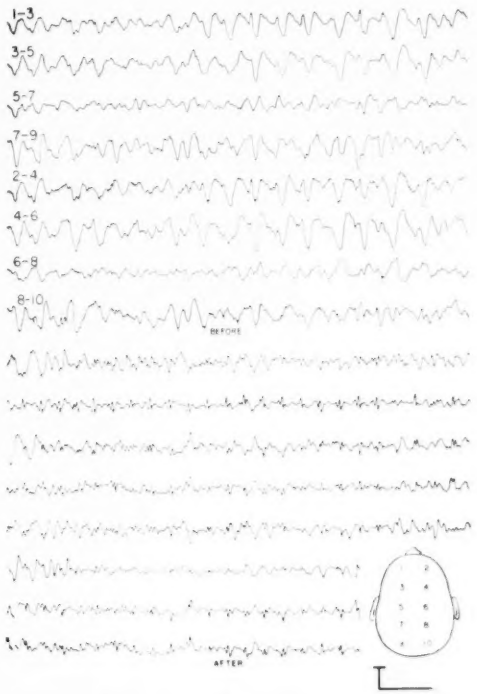


FIG. 1. ELECTROENCEPHALOGRAM BEFORE AND AFTER 5-HYDROXYTRYPTOPHAN (5-HTP) INFUSION
Calibration, 100 mV. and 1 second.

TABLE I

Clinical, metabolic and electroencephalographic response to 5-hydroxytryptophan (5-HTP) infusion

Patient, diagnosis	Av. 6 hr. urinary 5-HIAA*	I.V. 5-HTP	Recovery 5-HTP in 6 hrs.	Blood ammonia†	Blood 5-HT‡ Control	1 hr.	2 hr.	3 hr.	Electroencephalogram change after I.V. 5-HTP	Clinical observations	Comment
	mg.	*mg.*	*%*	*gamma/ml.*	*gamma/ml.*						
C. M. 12/6/56 Terminal hepatic coma	0.88	11.0		11/30 V, 1.55 A, 1.52 12/6 A, 0.99					Markedly diminished slow activity; prominent fast activity	None observed	Died 4 hours after 5-HTP
V. O. 12/28/56 Severe hepatic coma	0.13	10.2		12/28 A, 1.82 V, 0.59 JB, 1.06	0.14	0.10	0.10		Increased fast activity	Abdominal cramps; no inc. in peristalsis	Died 4 hours after 5-HTP
M. B. 1/17/57 Severe hepatic coma	1.37	10.9	46	1/17 A, 1.8 V, 1.1 JB, 1.7	0.18	0.11	0.19	0.12	Marked decrease in slow activity with increased frequency	Pulse inc. (16/min.) "Feel quiet and restful"	
M. C. 10/1/57 Mild hepatic coma	0.97	23.7	25	9/30 A, 1.44 V, 0.94	0.34	0.20	0.10		Moderate reduction of slow activity	Pulse inc. (24/min.)	
J. K. 12/23/57 Hodgkin's disease with mild hepatic coma	2.34 (per 24 hrs.)	40.4	46 (per 24 hrs.)	12/24 Fem. A, 0.66 Fem. V, 0.88	0.03	0.03	0.02	0.02	Reduction in slow activity with increased fast activity	None observed	On Predisone, 15 mg. t.i.d.; KCl, 1 Gm. t.i.d.; 2 ml. Thiomerin® prior to 5-HTP
					Platelet count 29,000/ml.						
Patients without hepatic insufficiency											
P. H. 2/19/57 Subdural hematoma	0.61	10.6	54		0.12	0.20	0.11	0.20	No change	Pulse inc. (26/min.); inc. peristalsis	
A. F. 2/7/57 Chronic congestive heart failure	0.85	10.2	36		0.21	0.45	0.55	0.49	No change	Abdominal burning and cramps; inc. peristalsis; BP inc. (120/70 to 160/100)	Died 7 hours after 5-HTP
W. C. 1/11/57 Normal	0.56	10.9	70		0.35	0.20	0.20	0.26	No change	"Tightness of stomach," "Can't get full breath," "Fullness in chest," "Feel cold." BP and pulse normal	
L. C. (W.F.—12) Epilepsy 3/28/57	0.37	12.1	48						No change	Inc. peristalsis; BP inc. (88/60 to 100/80)	

* Normal 24 hour urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA), 2 to 10 mg.
† Normal blood ammonia, less than 1 gamma per ml. arterial blood. Abbreviations are as follows: V, venous; A, arterial; JB, jugular bulb.
‡ Normal blood 5-hydroxytryptamine (serotonin), 0.1 to 0.3 gamma per ml.

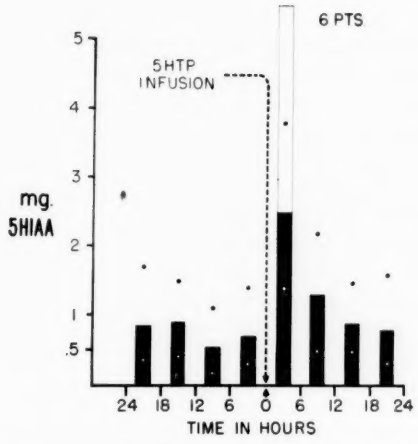


FIG. 2. INCREASED EXCRETION OF 5-HYDROXYINDOLEACETIC ACID (5-HIAA) FOLLOWING INTRAVENOUS 5-HYDROXYTRYPTOPHAN (5-HTP)

Dots represent high and low ranges. The bar is average excretion per six hour fractions.

hours in this patient and for about three hours in the remaining patients with hepatic insufficiency. In the three controls with abnormal electroencephalograms there was no change in the tracings on administration of 5-HTP, even though their tracings all showed markedly slowed activity bilaterally. In a normal individual, W. C., the administration of 5-HTP produced no changes in the electroencephalogram.

Figure 2 indicates the increased excretion of 5-HIAA following intravenously administered 5-HTP. Calculated on the assumption that only the l-isomer is metabolized, the average increased excretion in the first six hours represents a 46 per cent recovery of the administered dose of 5-HTP. If the excretion of the remaining 18 hours, in excess of the control period, is added to the first six hour excretion, a 52 per cent recovery is realized in 24 hours. The average 24 hour excretion of 5-HIAA was 3.1 mg. in the patients with hepatic coma and 2.4 mg. in the control patients. There was no significant difference in these values, which lie in the low normal range. In the case of Patient J. K., with liver failure secondary to extensive Hodgkin's disease involvement of the liver, spleen and retroperitoneal soft tissues, we had the opportunity to observe the effects of a diuretic on 5-HTP metabolism. Just prior to the administration of 40.4 mg. of 5-HTP, 2 ml. of mercaptomerin sodium was administered inadvertently. The urine resulting from the copious diuresis was collected in the indicated time intervals. In the first two hours (1,541 ml. urine volume) the 2.38 mg. of 5-HIAA excreted equaled the previous 24 hour 2.34 mg. of 5-HIAA. A single specimen containing the next 22 hours' excretion (4,780 ml.) of 8.08 mg. represented a 46 per cent recovery of administered 5-HTP in excess of the previous 24 hour control period. The diuresis apparently had no effect on the metabolism or excretion of 5-HIAA because these figures are similar to the data on all of the other patients in the study. The unusually low blood 5-HTP level probably reflects the thrombopenia secondary to nitrogen mustard therapy. There was no significant change in platelet serotonin levels.

Figure 3 indicates the average change in platelet 5-HT content during the three hours following 5-HTP injection. Although there is a terminal rise, this is just outside our range of error for 5-HT measurement. Such equivocal results may be explained by our low (0.1 to 0.3 mg. per Kg.) 5-HTP dosage as compared with animal experiments (11) where 400 to 1,000 times (60 to 150 mg. per Kg.) as much 5-HTP was given, produc-

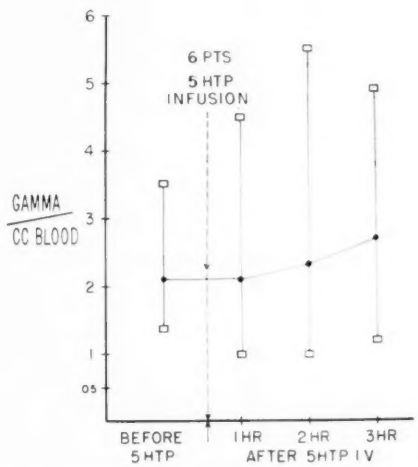


FIG. 3. RESPONSE OF BLOOD (PLATELET) 5-HYDROXYTRYPTAMINE TO 5-HYDROXYTRPTOPHAN (5-HTP) INFUSION

Blocks represent ranges; dots, average.

ing a fourfold increase in blood 5-HT associated with markedly toxic central nervous system disturbances. These data confirm the statement made by Davidson, Sjoerdsma, Loomis and Udenfriend (12) that this dosage level of 5-HTP produced no effect on the blood serotonin in animals.

CLINICAL OBSERVATIONS

In the patients with hepatic insufficiency there was no change in tremors, mental confusion or reflex activity during the administration of 5-HTP, or for a three hour follow-up period. Patient

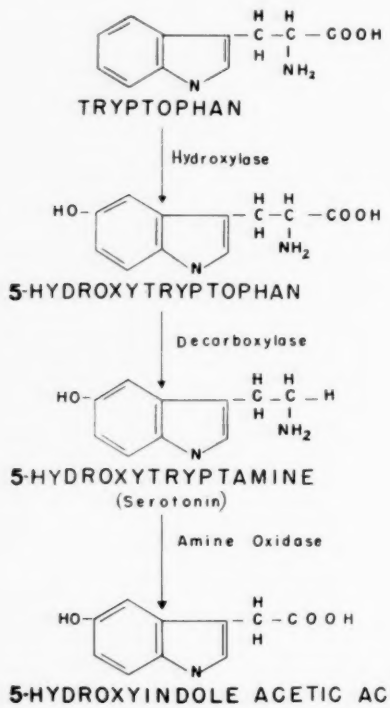


FIG. 4. METABOLIC PATHWAY OF 5-HYDROXYTRYPTAMINE (5-HT)

M. B., however, stated that she felt "quiet and restful" but demonstrated no improvement in her restlessness. Three patients (M. B., M. C. and P. H.) had significant increases in pulse rate which subsided following the 30 minute injection period. Two patients (A. F. and L. C.) demonstrated significant (average, 26/25) rises in blood pressure which subsided within 5 to 10 minutes after cessation of I. V. 5-HTP. The only consistent observation was that of increased peristalsis, observed in four patients (V. O., P. H., A. F. and L. C.), with three of these complaining of abdominal discomfort (cramps and burning). Interestingly, J. K., who received four times the usual dose, displayed none of these effects. 5-HTP injection was not felt to contribute to the demise of the three patients dying three to seven hours following injection, since all three patients were extremely ill and were chosen for this reason as initial subjects.

DISCUSSION

Severe liver failure is frequently associated with serious disturbances of consciousness. This study was undertaken to determine whether the liver played any role in metabolism of serotonin and whether such metabolism might be deranged by liver failure. Figure 4 indicates the normal pathway of serotonin formation with its breakdown to 5-hydroxyindoleacetic acid. All of these steps have been demonstrated in animal tissues except the conversion of tryptophan into 5-hydroxytryptophan (13). As yet, the site of formation of this intermediate has not been demonstrated. It is quite clear from animal experiments that parentally injected 5-hydroxytryptamine is not able to enter the brain, whereas 5-hydroxytryptophan does enter the brain and causes a marked increase in the level of serotonin in that organ (11). It is most likely that the source of serotonin in the brain is 5-HTP formed elsewhere in the body. The conversion of tryptophan to 5-HTP is the type of reaction which is known to occur in the liver, the site of most hydroxylations. If the formation of 5-HTP were a function of the liver, one could expect that in severe liver failure there might be a defect in serotonin metabolism. The average 24 hour excretion of 5-HIAA for both groups (hepatic coma, 3.2 mg.; controls, 2.4 mg.) is in the low normal range when compared with 21 normal adults (average, 4.7 mg.; range, 2.3 to 9.7 mg. per 24 hours). The experiments herein reported do show that in severe liver disease 5-HTP can be converted to 5-HIAA, presumably by going through the 5-HT step.

The fact that only patients with liver disease show an effect of 5-HTP upon the electroen-

cephalogram suggests the possibility that within the brain a deficiency of serotonin exists, resulting from the inability of the patient with hepatic coma to convert tryptophan to 5-HTP. According to these results it appears as if there is some defect in serotonin formation in liver disease prior to 5-HTP (see Figure 4). Another possibility is that the observed electroencephalogram changes following infused 5-HTP simply represent a pharmacologic effect upon the brain akin to changes recorded in the gastrointestinal tract. Were this the case, however, it should have appeared in the controls.

SUMMARY

5-Hydroxytryptophan (5-HTP), 10 to 40 mg., was given intravenously to nine patients while electroencephalograms were recorded. In all five patients with hepatic coma, the slow activity diminished and the fast activity increased, altering the record in the direction of normal. In three patients with coma, not due to liver failure, and in one normal individual, there were no alterations in the electroencephalogram. Twenty-four hour urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA) accounted for 52 per cent of the 5-hydroxy-L-tryptophan administered. No definite defect was observed in the ability of patients with liver disease to metabolize 5-hydroxytryptophan as compared with a control group.

At the dosage levels utilized, infused 5-hydroxytryptophan produced no effect on blood serotonin.

In patients with hepatic insufficiency, there was no change in tremors, mental confusion or reflex activity. Abdominal discomfort and increased peristalsis were frequent.

ACKNOWLEDGMENTS

We are especially indebted to Miss Anne Rider for her careful technical assistance. We gratefully acknowledge the aid of Mrs. Howard Robertson and Miss Jennie Ann Gray who recorded the electroencephalograms. We also wish to thank Dr. D. A. Shepard, the Upjohn Co., and Dr. George H. Berryman, Abbott Laboratories, for supplying the 5-hydroxytryptamine (serotonin) used in this study.

REFERENCES

1. Borges, F. J., and Bessman, S. P. Serotonin. Ann. intern. Med. 1957, **46**, 425.
2. Erspamer, V. Pharmacology of indolealkylamines. Pharmacol. Rev. 1954, **6**, 425.
3. Page, I. H. Serotonin (5-hydroxytryptamine). Physiol. Rev. 1954, **34**, 563.
4. Page, I. H. Serotonin (5-hydroxytryptamine); the last four years. Physiol. Rev. 1958, **38**, 277.
5. Bogdanski, D. F., Weissbach, H., and Udenfriend, S. The distribution of serotonin, 5-hydroxytryptophan decarboxylase, and monoamine oxidase in brain. J. Neurochem. 1957, **1**, 272.
6. Haverback, B., Jr., Sjoerdsma, A., and Terry, L. L. Urinary excretion of the serotonin metabolite, 5-hydroxyindoleacetic acid, in various clinical conditions. New Engl. J. Med. 1956, **255**, 270.
7. Bessman, S. P., Merlis, J. K., and Borges, F. Effect of 5-hydroxytryptophane upon electroencephalogram in hepatic coma. Proc. Soc. exp. Biol. (N. Y.) 1957, **95**, 502.
8. Udenfriend, S., Titus, E., and Weissbach, H. Identification of 5-hydroxy-3-indoleacetic acid in normal urine and method for its assay. J. biol. Chem. 1955, **216**, 499.
9. Udenfriend, S., Weissbach, H., and Clark, C. T. The estimations of 5-hydroxytryptamine (serotonin) in biological tissues. J. biol. Chem. 1955, **215**, 337.
10. Seligson, D., and Seligson, H. A microdiffusion method for the determination of nitrogen liberated as ammonia. J. Lab. clin. Med. 1951, **38**, 324.
11. Udenfriend, S., Weissbach, H., and Bogdanski, D. F. Increase in tissue serotonin following administration of its precursor 5-hydroxytryptophan. J. biol. Chem. 1957, **224**, 803.
12. Davidson, J., Sjoerdsma, A., Loomis, L. N., and Udenfriend, S. Studies with the serotonin precursor, 5-hydroxytryptophan, in experimental animals and man. J. clin. Invest. 1957, **36**, 1594.
13. Udenfriend, S., Titus, E., Weissbach, H., and Peterson, R. E. Biogenesis and metabolism of 5-hydroxyindole compounds. J. biol. Chem. 1956, **219**, 335.

CALCULATION OF INTRACELLULAR pH FROM THE DISTRIBUTION OF 5,5-DIMETHYL-2,4-OXAZOLIDINEDIONE (DMO). APPLICATION TO SKELETAL MUSCLE OF THE DOG*

By WILLIAM J. WADDELL and THOMAS C. BUTLER

(From the Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, N. C.)

(Submitted for publication October 6, 1958; accepted January 2, 1959)

Estimations of hydrogen ion activities in the interior of cells have been attempted through a number of different experimental approaches. The methods that have been employed are of several general types: measurements on preparations of broken cells, measurements on fluid withdrawn from cells, observations with visible indicators, measurements with micro-electrodes introduced into cells, measurements of the intracellular and extracellular concentrations of weak organic acids and bases, and calculation of the balance of ionic charges. The last named method has been proposed by Conway (1). The other methods are discussed in the review of Caldwell (2), which contains a tabulation of the results of measurements on various types of cells with different experimental methods. Some of the methods that have been used are of quite limited applicability, and most of the values arrived at by any of the methods are subject to question.

Potentially, the method utilizing the distribution of weak organic acids and bases has a wider range of applicability than the other methods, and it is not subject to some of the theoretical objections that can be raised to other methods. There is a great deal of evidence that membranes of animal and plant cells in general are characterized by free permeability to the undissociated forms of weak organic acids and bases and by impermeability or apparent impermeability to the ionic forms. If the pH of the interior of a cell differs from that of the external medium in contact with it, there will be a corresponding difference in the concentration of an organic ion. The assumption that the undissociated form of an acid or base is in equal concentration on the two sides of the cellular membrane permits the calculation of intracellular pH from measurements of extracellular pH and the extracellular and intracellular concentrations of the acid or base. In most of the work that has hitherto been done based on this principle, carbon dioxide has been the compound used as the indicator. It is assumed that the cellular membrane is permeable to carbon dioxide and that the tension of carbon dioxide inside the cell is equal to that measured in an external phase in equilibrium with the cell. The total carbon dioxide released from the cells after addition of acid is determined and is assumed to come from dissolved carbon dioxide, carbonic acid and bicarbonate ion. These data are sufficient for calculation of the intracellular concentration of bicarbonate ion and of the intracellular pH. The pK_1' of carbonic acid is such that its ionization should furnish a sensitive measure of intracellular pH. However, the validity of pH measurements made with the carbon dioxide method has been questioned. Conway and Fearon (3) concluded from their experiments that a large part of the carbon dioxide released from muscle cells by acid is derived from acid-labile material other than bicarbonate. If this should be so, the pH values calculated from the assumption that all acid-labile carbon dioxide comes from bicarbonate would be seriously in error.

In the course of a study of the tissue distribution of 5,5-dimethyl-2,4-oxazolidinedione ("DMO"), the product of metabolic N-demethylation of the antiepileptic agent, trimethadione (Tridione®) (4), we have found that the attributes desirable in a compound to be used for the measurement of intracellular pH are combined to an almost ideal degree in DMO. These properties will be dis-

* This investigation was supported in part by a research grant (B-384) from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service.

cussed in detail below. The advantages of DMO over carbon dioxide are in the facility of analytical determination and in the absence of any question as to the chemical identity of the material determined by the analytical procedure. The present report comprises a theoretical consideration of the estimation of intracellular pH from the distribution of acids and bases and an application of the DMO method to the estimation of intracellular pH of skeletal muscle of the dog under various conditions. We have been particularly interested in muscle because other methods of estimating intracellular pH have been applied more extensively to muscle than to other mammalian tissues, and because changes of pH in muscle are of great importance in buffering on account of the large bulk of the tissue. With muscle, unlike some other tissues, it is possible to take several successive samples from a single animal during the course of an experiment without disturbing the conditions. Furthermore, there is evidence that the chloride of muscle is largely extracellular (5), and determination of the chloride space affords a method of measuring extracellular water that is convenient and probably sufficiently accurate for this purpose. Extracellular water of some other tissues cannot be estimated with as great ease or certainty.

THEORETICAL CONSIDERATIONS

Mathematical formulations. We shall consider a tissue containing intracellular water of volume V_i and extracellular water of volume V_e, the intracellular phase being contained within membranes freely permeable to the undissociated form of a weak organic acid or base (to be called the "indicator compound"). V_e can be derived from the distribution of a substance confined to extracellular water, and V_i from the difference between V_e and total tissue water. Neither the intracellular nor the extracellular tissue phase is individually accessible for analysis, but the concentration, C_t, of the indicator compound in the water of the entire tissue can be determined. By determinations in plasma with corrections for the Donnan equilibria, the pH and the concentration of the indicator compound in the extracellular tissue water (pH_e and C_e, respectively) can be derived. It is assumed that the aqueous phases are homogeneous and that the indicator compound is fully equilibrated, the concentration of the undissociated form being equal in intracellular water, extracellular tissue water and plasma water. If this equality of concentration of the undissociated form is maintained regardless of pH differences that may exist between the two sides of the cellular membrane, it is immaterial to the theory through what mechanism the pH gradient is brought about or whether the membrane is considered to be truly impermeable to the ionic form of the indicator compound. The apparent ionization constant and the activity coefficient of the ion are assumed to be equal in all the aqueous phases. Then, if the indicator compound is a weak organic acid with apparent ionization exponent, pK′, the pH of the intracellular water, pH_i, can be expressed in terms of measurable quantities or values mathematically derived from measurable quantities:

$$pH_i = pK' + \log\left\{\left[\frac{C_t}{C_e}\left(1 + \frac{V_e}{V_i}\right) - \frac{V_e}{V_i}\right] \times \left[10^{(pH_e - pK')} + 1\right] - 1\right\}. \quad 1)$$

If the indicator compound is a weak organic base with apparent acid ionization exponent, pK_a',

$$pH_i = pK_a' - \log\left\{\left[\frac{C_t}{C_e}\left(1 + \frac{V_e}{V_i}\right) - \frac{V_e}{V_i}\right] \times \left[10^{(pK_a' - pH_e)} + 1\right] - 1\right\}. \quad 2)$$

The partial derivative of the ratio (C_t/C_e) with respect to pH_i is an index of the precision with which pH_i can be calculated from measured concentrations of the indicator compound. For an acid,

$$\frac{\partial(C_t/C_e)}{\partial(pH_i)} = \ln 10\left(\frac{V_i}{V_i + V_e}\right)\left(\frac{10^{pH_i}}{10^{pH_e} + 10^{pK'}}\right). \quad 3)$$

For a base,

$$\frac{\partial(C_t/C_e)}{\partial(pH_i)} = -\ln 10\left(\frac{V_i}{V_i + V_e}\right)\left(\frac{10^{-pH_i}}{10^{-pH_e} + 10^{-pK_a'}}\right). \quad 4)$$

These partial derivatives as functions of the ionization exponents of acids and bases are shown in Figures 1 and 2, respectively. In these figures, pH_e is assumed to be 7.4 and pH_i 7.0 (approximately the pH_i of muscle as found in this study). The functions are without maxima, increasing to approach asymptotic limits with decreasing values of pK′ for acids and increasing values of pK_a' for bases. If pH_i is less than pH_e, as is assumed in these figures, the limiting value is greater for a base than for an acid. The stronger an acid or a base, the more sensitive an indicator it is of intracellular pH.[1] If intracellular pH is less than plasma pH, a base of sufficient strength can be a more sensitive indicator than any acid.

For an acid of the strength of DMO and for the values of pH_i, pH_e, V_i, and V_e assumed in Figures 1 and 2, an error of 0.01 unit in the calculated value of pH_i would be introduced by an error of 1.4 per cent in the ratio (C_t/C_e) or by an error of 7.7 per cent in V_e. If the ionic strength of intracellular water should differ from that of extracellular water by as much as 0.05, the pK′

[1] This theory is not applicable to compounds that are completely dissociated such as sulfonic acids and quaternary nitrogen compounds.

values in the two phases as predicted by the Debye-Hückel theory would differ by as much as 0.03 unit, and an error of that magnitude would be introduced in the calculated value of pH_i.

The assumption made above that all aqueous phases are homogeneous is undoubtedly not altogether valid, particularly with respect to the intracellular water. If the cell interior is nonuniform with respect to pH, or if the cells in the mass of tissue analyzed differ among themselves, the value calculated for intracellular pH is not a mean value in the mathematical sense. It has been termed an "aggregate" pH by Irving, Foster and Ferguson (6) and an "overall" pH by Conway and Downey (7). Despite the fact that the calculated intracellular pH may not have a simple mathematical significance, it is a value intermediate between the values for the regions of highest and lowest pH in the intracellular water of the tissue and thus furnishes some information, however limited, concerning the conditions in the cells. Of even greater interest than the calculated pH for the normal cell are the changes that this value undergoes under physiological and pathological conditions, for these changes can be interpreted in terms of the buffering capacity of cells and the gain or loss of hydrogen ions by the cells.

Properties to be desired in a compound to be used for measurement of intracellular pH in animal tissues. To be an adequately sensitive indicator of intracellular pH, the compound must be a sufficiently strong acid or base. An acid with pK′ less than about 7.0 or a base with pK_a' greater than about 7.0 will be satisfactory for the physiological values assumed in Figures 1 and 2. In concentrations that are analytically determinable the compound should produce no pronounced pharmacological effects,

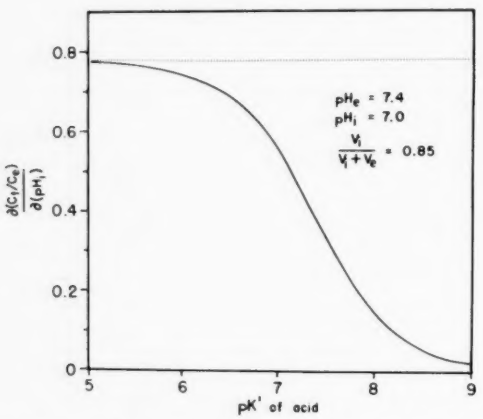


FIG. 1. THE PARTIAL DERIVATIVE OF THE RATIO OF CONCENTRATION IN TOTAL TISSUE WATER (C_T) TO CONCENTRATION IN EXTRACELLULAR TISSUE WATER (C_E) WITH RESPECT TO INTRACELLULAR PH (pH_i) AS A FUNCTION OF THE APPARENT IONIZATION EXPONENT FOR WEAK ORGANIC ACIDS

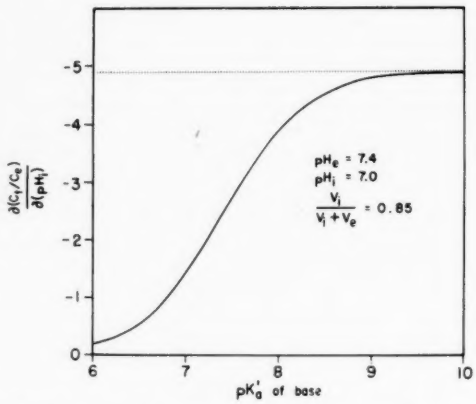


FIG. 2. THE PARTIAL DERIVATIVE OF THE RATIO OF CONCENTRATION IN TOTAL TISSUE WATER (C_T) TO CONCENTRATION IN EXTRACELLULAR TISSUE WATER (C_E) WITH RESPECT TO INTRACELLULAR PH (pH_i) AS A FUNCTION OF THE APPARENT ACID IONIZATION EXPONENT FOR WEAK ORGANIC BASES

toxic or otherwise, and should not of itself affect acid-base equilibria inside or outside of cells. The compound should not interact with plasma proteins or with any constituents of the cell to a degree that would significantly affect its distribution. It should not be localized to a significant extent in nonaqueous phases of cells or plasma. Although not essential, it is desirable that the compound be slowly eliminated so that after a single dose the plasma concentration will change very little during the period required for equilibration of the compound between plasma and tissues. Finally, an analytical method must be available that is specific and sufficiently sensitive for concentrations that have no disturbing effect.

Properties of DMO. The compound is an acid with pK′ of 6.13 at 37° C. and ionic strength of 0.16.[2] As shown in Figure 1, this value of pK′ gives almost as sensitive an indication of intracellular pH as is theoretically possible for an acid. We have used plasma concentrations of DMO no higher than 250 μg. per ml. in dogs for measurement of muscle pH. There are no

[2] The pK′ of DMO has not been measured in protein solutions. The pK_1' of carbonic acid, which is nearly equal to that of DMO, is not affected to a great extent by the presence of protein in solutions of egg albumin, in muscle homogenates, or in erythrocyte hemolysates (8, 9). The assumption that the pK′ of DMO is equal in intracellular water, interstitial fluid, and plasma probably does not involve a serious error in the estimation of intracellular pH. The error in the calculated value of pH_i contributed by an error in the value of pK′ for DMO in intracellular water is equal to the latter error and is constant. The magnitude of a *change* in pH_i occurring under experimental conditions would be measured without error.

grossly observable pharmacological effects at this level, while the concentrations in plasma and muscle are high enough to permit analytical measurements of sufficient accuracy. Patients receiving trimethadione may have plasma concentrations of DMO as high as 1 mg. per ml. without any evidence of toxic effect. DMO is not bound to plasma albumin (10). As reported in this paper, DMO is not bound to an homogenate of dog muscle to a measurable degree. The concentration of DMO in fat is very low relative to that in plasma, as evidenced by the finding that the apparent volume of distribution of DMO in omental fat of the dog is nearly equal to the chloride space and to the total volume of water of the tissue. It is unlikely that DMO is contained in lipid phases in the cell in proportions of any importance. There is no reason, then, to believe that the distribution of DMO is significantly affected by any interactions or by any localizations in nonaqueous phases that would invalidate the calculation of intracellular pH. DMO is among the most persistent drugs that have been studied. It is not metabolized, and excretion is very slow in acid urine (10). In the normal dog after equilibration, the hourly decline in plasma concentration is only about 1 to 2 per cent. For measurements of tissue pH, a single dose of DMO can be administered to an animal, and the equilibration of tissues with plasma is not complicated by a rapidly falling plasma concentration. DMO is determinable in plasma and tissues by ultraviolet spectrophotometric methods of adequate sensitivity and of demonstrated specificity.

METHODS AND MATERIALS

Synthesis of DMO. Ethyl α-hydroxyisobutyrate was condensed with urea in the presence of sodium ethylate by the method of Stoughton (11). DMO melts at 78 to 79° C.

Experiments on dogs. In some early studies of the pH of tissues under normal conditions, dogs were anesthetized with ether or were killed by intravenous injection of air without anesthesia. In all of the experiments to be described in this report in which the effects of acidosis and alkalosis were studied, the dogs were anesthetized with 125 mg. per Kg. of phenobarbital adminstered intravenously as a solution of the sodium salt. DMO was given intravenously in saline solution in a dose of 100 mg. per Kg. at least an hour before the first muscle sample was taken. This time has been found to be more than sufficient for equilibration of DMO between plasma and muscle. Both renal pedicles were ligated tightly so as to occlude the renal vessels and the ureters. CO_2–O_2 mixtures were administered with an anesthetic gas machine. Overventilation was produced with a respiration pump. HCl was administered by slow intravenous infusion of a 0.30 N solution. $NaHCO_3$ was given intravenously in 0.30 or 0.95 M solution. Samples were taken from the muscles of the thighs. The muscle samples were blotted with cotton gauze, and all visible fat and connective tissue were trimmed away. Blood samples were taken from the femoral veins, about 3 mg. of potassium oxalate per ml. of blood being used as anticoagulant. Blood used for determination of pH and CO_2 was handled with strict anaerobic precautions.

Total water of muscle and plasma was determined by drying weighed samples in an oven at 120° C. for at least 12 hours. There was found to be so little variation in the water content of plasma that this determination was omitted for some samples, and the value of 0.93 Gm. per Gm. was used in the calculations.

Determination of DMO. For plasma from unanesthetized dogs or dogs anesthetized with ether, the method of Butler (12) was used. For plasma from dogs anesthetized with phenobarbital the method was modified in that the extract in the buffer of pH 9.0 was divided into two parts and the subsequent procedures were carried out as described below for the method for muscle. For muscle, a sample of 5 to 10 Gm. was homogenized with 10 ml. of distilled water with the homogenizer described in a previous study (13). A weighed sample of 1 to 3 Gm. of the homogenate was transferred to a glass stoppered tube, in which it was shaken with 4 ml. of 5 M NaH_2PO_4 and 25 ml. of redistilled reagent grade absolute ethyl ether previously saturated with water. After centrifugation, 20 ml. of the ether phase was removed and extracted with 5 ml. of the borate buffer of pH 9.0 as in the original plasma method. Of the buffer extract, 2 ml. portions were transferred to each of two tubes. To one was added 2 ml. of the buffer of pH 9.0 and to the other 2 ml. of 0.1 N HCl. Absorbancies of both preparations were measured at 215 and 220 mμ, the blank for the first being the buffer, and for the second, equal volumes of buffer and 0.1 N HCl. The absorbancies at pH 9.0 were corrected by substracting the corresponding absorbancies in acid solution. The concentration of DMO was calculated from the difference between the corrected absorbancy at 215 mμ and that at 220 mμ. When this method was applied to muscle from dogs that had had no drug, the calculated concentrations of DMO were between 1 and 2 μg. per Gm. When DMO was added to normal muscle in concentrations of 50 to 100 μg. per Gm., the recoveries were 96 to 104 per cent. Since the blank values are low relative to the concentrations to be determined, and since DMO is not metabolically altered in the body, the material determined as DMO in muscle can be considered to consist almost entirely of that compound. Ether and phenobarbital in anesthetic concentrations do not interfere with this method for muscle.

Determination of chloride. For plasma the method of Van Slyke and Hiller (14) was used. For muscle a sample of about 15 Gm. of the same homogenate prepared for the DMO determination was weighed into a glass stoppered graduated centrifuge tube. To the tube were added 5 ml. of a solution containing 150 Gm. of $Na_2WO_4 \cdot 2H_2O$ and 250 ml. of concentrated H_3PO_4 per L., about 0.5 Gm. of $AgIO_3$, and sufficient distilled water to make the total volume to the nearest multiple of 5 ml. The tube was shaken for 1 hr. and centrifuged. The supernatant liquid was filtered through filter paper, and a measured volume of the filtrate was titrated with thiosulfate as in the method for plasma.

Determination of plasma CO_2. Blood was centrifuged under mineral oil and the plasma CO_2 was determined by the method of Van Slyke and Neill (15).

Measurement of pH. The pH of blood was measured at 37° C. by the procedure described in another report (13).

Calculations. It was assumed that Donnan equilibria prevail between plasma and extracellular tissue water for the hydrogen ion, the chloride ion, and the DMO ion. The pH of the extracellular tissue water, pH_e, was assumed to be 0.02 unit higher than that of plasma and the concentrations of chloride and DMO ions in extracellular tissue water to be 1.05 times the corresponding concentrations in plasma water.[3] On the assumption that all chloride in muscle is extracellular, the volume of extracellular water, V_e, as a proportion of total tissue weight was calculated by dividing the chloride concentration in tissue by the concentration in extracellular tissue water. The volume of intracellular water, V_i, as a proportion of total tissue weight was derived by substracting V_e from the total proportion of water in the tissue. The concentration of DMO in extracellular tissue water, C_e, was calculated from the assumption that the concentration of the ion differes from that in plasma water by the Donnan ratio and that the concentration of undissociated form is the same in the water of the two phases. The CO_2 tension of plasma was calculated from the total CO_2 content of plasma and the pH of blood by use of the Henderson-Hasselbalch equation, the pK′ of carbonic acid being taken as 6.10 and the solubility coefficient, α, of CO_2 as 0.53.

Binding of DMO to muscle homogenate. A sample of 4 Gm. of dog muscle was homogenized with 15 ml. of 0.2 M phosphate buffer of pH 7.0. With the procedure described in an earlier report (13), this homogenate was dialyzed in a cellophane bag against a large volume of the buffer containing 100 μg. per ml. of DMO. The contents of the bag and of the outer chamber were analyzed for DMO by the method described above for muscle.

RESULTS

Binding of DMO to muscle homogenate

When the homogenate of dog muscle described under Methods was dialyzed at 37° C. against a buffer of pH 7.0 containing 100 μg. per ml. of DMO, there was no measurable binding of DMO to the homogenate.

Intracellular pH of dog muscle

In 11 dogs that had received no treatment designed to disturb acid-base equilibria, of which seven had been anesthetized with 125 mg. per Kg. of phenobarbital, two anesthetized with ether, and two killed by air embolism, the calculated intracellular pH values for muscle were all in the range of 6.96 to 7.10 with an average of 7.04. This total range is only of the order of magnitude of perhaps twice the expected experimental error, and no effect of anesthesia on pH was apparent.

The effects of various treatments altering the pH or the pCO_2 of plasma were studied. In the experiments described below, two different treatments were applied successively to each dog. The combinations of treatments were chosen with the purpose of furnishing controlled comparisons of changes in pH and pCO_2 under various conditions. Table I shows the analytical data and derived values from which the intracellular pH is calculated. In addition to the experiments described in Table I, there were eight other satisfactory experiments in which at least one of these same treatments was studied in each dog. In all of these experiments, the effects of the treatments were essentially the same as in the corresponding experiments of Table I.

In Experiment 1 of Table I a dog inhaled 30 per cent carbon dioxide, and sodium bicarbonate was later administered intravenously while the carbon dioxide inhalation was continued. As the pCO_2 of venous plasma rose, the pH of venous blood fell by 0.43 unit and the calculated intracellular pH of muscle fell by 0.28 unit. Administration of 20 mM per Kg. of sodium bicarbonate restored the blood pH to its original value, but the plasma pCO_2 was increased somewhat and the muscle pH was further lowered to 6.61.

In Experiment 2 the same two treatments were given in reverse order. An intravenous dose of 10 mM per Kg. of sodium bicarbonate raised the pH of blood by 0.20 unit and the intracellular pH of muscle by 0.11 unit without significant effect

[3] The value of the Donnan ratio varies with the pH of plasma as well as with the composition of plasma with respect to protein and inorganic ions. The value used here is a theoretical value calculated for protein-free extracellular tissue water equilibrated with plasma containing 160 mM per L. of anions other than protein and 17 mM per L. of base bound to protein. Experimentally determined ratios for different ions in different plasma ultrafiltrates and transudates sometimes deviate significantly from theoretical values (5). There is no direct knowledge of the actual values of the ratios for the hydrogen, chloride, and DMO ions in interstitial fluid of muscle. The error in the estimation of the Donnan ratio probably does not introduce any large error in the calculation of intracellular pH.

TABLE I

Effects of various procedures altering acid-base equilibria in dogs. Analytical data and derived values used in the calculation of intracellular pH of muscle

Time	Measured Values							Calculated Values						
	Muscle			Plasma										
	DMO	Cl^-	H_2O	DMO	Cl^-	CO_2	pH	C_t	C_e	V_e	V_i	pH_e	pCO_2	pH_i
Min.	μg/Gm.	mEq./Kg.	Gm./Gm.	μg/Gm.	mEq./Kg.	mM/L.		μg/Gm.	μg/Gm.				mm Hg	
Experiment 1. Female dog, 4 Kg.														
0	99	24.7	0.75	192	114	25.8	7.35	132	217	0.19	0.56	7.37	44	7.02
50–210	Inhalation of 30 % CO_2.													
120	109	24.6	0.76	168	122	30.0	6.92	143	190	0.18	0.58	6.94	126	6.74
135–185	Intravenous infusion of $NaHCO_3$, 20 mM/Kg. (88 ml. of 0.95 M)													
205	82	19.8	0.76	226	87	92.5	7.35	108	255	0.20	0.56	7.37	157	6.61
Experiment 2. Male dog, 15 Kg.														
0	94	13.1	0.76	190	111	23.6	7.34	124	215	0.10	0.66	7.36	41	7.04
30–60	Intravenous infusion of $NaHCO_3$, 10 mM/Kg. (145 ml. of 0.95 M)													
200	83	11.6	0.75	202	104	38.5	7.54	111	228	0.10	0.65	7.56	43	7.15
255–375	Inhalation of 34 % CO_2.													
370	75	11.4	0.76	192	105	36.2	7.32	99	217	0.10	0.66	7.34	66	6.89
375–425	Inhalation of 50% CO_2.													
420	83	12.6	0.75	185	100	39.8	7.10	111	209	0.11	0.64	7.12	116	6.71
Experiment 3. Female dog, 9 Kg.														
0	94	13.5	0.76	184	112	24.0	7.35	124	208	0.11	0.65	7.37	41	7.07
30–290	Overventilation													
110	91	20.4	0.76	204	114	17.0	7.56	120	230	0.16	0.60	7.58	18	7.15
140–225	Intravenous infusion of HCl, 6 mM/Kg. (182 ml. of 0.3 N)													
285	81	14.7	0.77	155	129	9.4	7.30	105	175	0.10	0.67	7.32	18	7.02
Experiment 4. Male dog, 16 Kg.														
0	90	13.1	0.75	190	112	20.9	7.35	120	215	0.10	0.65	7.37	36	7.03
30–240	Intravenous infusion of HCl, 7 mM/Kg. (372 ml. of 0.3 N)													
300	99	17.9	0.75	141	122	12.7	6.99	132	159	0.13	0.62	7.01	46	6.90
315–355	Intravenous infusion of $NaHCO_3$, 3.75 mM/Kg. (200 ml. of 0.3 M)													
425	92	16.6	0.74	168	116	14.9	7.19	124	190	0.13	0.61	7.21	36	6.95
455–475	Intravenous infusion of $NaHCO_3$, 3.25 mM/Kg. (172 ml. of 0.3 M)													
555	81	15.1	0.75	166	117	14.2	7.25	108	188	0.11	0.64	7.27	30	6.94

on pCO_2 of plasma. Inhalation of 34 per cent carbon dioxide lowered the blood pH to normal while raising the plasma pCO_2 and lowering the pH of muscle. Further increase in the inhaled concentration of carbon dioxide to 50 per cent caused additional changes in the same direction, the muscle pH finally reaching a value of 6.71.

In Experiment 3 the pCO_2 of plasma was lowered by overventilation with a respiration pump. Intracellular pH of muscle rose by 0.08 unit as the pCO_2 of plasma fell and the pH of blood rose. Subsequently, while overventilation was continued, 6 mM per Kg. of hydrochloric acid was infused intravenously. The muscle pH as well as the blood pH fell somewhat below their initial levels without significant change in the plasma pCO_2.

In Experiment 4 the initial treatment was an intravenous infusion of 7 mM per Kg. of hydrochloric acid. There was only a slight rise of plasma pCO_2, which may have been due to depression of respiration consequent to deepening of phenobarbital anesthesia in acidosis (13). While the blood pH fell by 0.36 unit, the intracellular muscle pH fell by only 0.13 unit. After the injection of an equivalent amount of sodium bicarbonate, the blood pH rose by 0.26 unit while

the muscle pH rose by only 0.04 unit, an amount that probably does not exceed the experimental error. In two other dogs in which acidosis was produced by intravenous infusion of 10 mM per Kg. of hydrochloric acid, the intracellular pH of muscle did not fall below 6.90 even though the blood pH in one dog fell to 6.84.

DISCUSSION

In these experiments the largest changes in the intracellular pH of muscle are those brought about by increases in the carbon dioxide tension of blood. In severe metabolic acidosis with normal carbon dioxide tension the intracellular pH of muscle is not lowered to the extent that it is in conditions of elevated carbon dioxide tension. If the carbon dioxide tension of plasma is abnormally high, the pH of muscle is abnormally low even though the pH of blood may be normal. Both metabolic and respiratory alkalosis may cause some increase in intracellular pH of muscle, but the increases that can be produced by any treatment are small by comparison with the decreases resulting from increased tension of carbon dioxide.

The effect of carbon dioxide tension on the intracellular pH of muscle is to be expected if the muscle fibers are freely permeable to carbon dioxide, as cellular membranes in general have been found to be. That greater effects on the pH of muscle can be produced by raising the carbon dioxide tension above normal than by lowering it below normal is in accordance with the form of the carbon dioxide dissociation curve of muscle as described by Irving and co-workers (6). The study of Swan and Pitts (16) indicates that hydrochloric acid administered intravenously to nephrectomized dogs is neutralized in part by loss of intracellular sodium and potassium ions from tissues in exchange for hydrogen ions from extracellular water. Experiments of the same type carried out by Schwartz, Ørning and Porter (17) in dogs with intact kidneys led to similar conclusions. Tobin (18) found that intravenous hydrochloric acid in nephrectomized cats caused a shift of sodium and potassium into the extracellular space and a loss of sodium and potassium from muscle. However, in Tobin's experiments the average values of intracellular muscle pH as measured by the carbon dioxide method did not change after cats had received doses of 3.5 to 9.6 mM per Kg. of hydrochloric acid. Wallace and Hastings (19) did not find significant changes of muscle pH as measured by the carbon dioxide method after intravenous doses of 1 mM per Kg. of hydrochloric acid to cats. In our experiments in dogs the intracellular pH of muscle was significantly lowered by intravenous administration of hydrochloric acid, but the fall of muscle pH was no more than about 0.15 unit even in severe metabolic acidosis. The ionic exchanges that have been postulated to occur in metabolic acidosis apparently can operate in muscle only to the extent that intracellular buffering is sufficient to prevent any large change of intracellular pH. Studies of the intravenous administration of sodium bicarbonate to human subjects by Singer and associates (20) and to nephrectomized dogs by Swan, Axelrod, Seip and Pitts (21) have been interpreted as indicating the disappearance of about a quarter of the injected sodium from the extracellular space. One possible mechanism that would produce this effect would be the entry of sodium ions into cells either accompanied by bicarbonate ions or in exchange for hydrogen ions. Either process would tend to raise the intracellular pH. Wallace and Hastings (19) found no significant elevation of intracellular pH of muscle of cats in response to sodium bicarbonate injection. In our experiments the effects of sodium bicarbonate administration on muscle pH were quite small. The extent to which sodium ions may pass into muscle cells during the period of these acute experiments cannot be estimated from the present data. Intracellular buffering and possibly other mechanisms such as the intracellular production of organic acids may suffice to counteract the effect of entry of sodium to the extent that any change of pH may be so small as even to be difficult to detect. If the administration of sodium bicarbonate is accompanied by an increase in the carbon dioxide tension of blood, the result may actually be a decrease in the intracellular pH of muscle.

The values for intracellular pH of frog and mammalian muscle under normal conditions as measured by other workers with the carbon dioxide method have for the most part been in the range of 6.9 to 7.3. References to the earlier reports of the use of the carbon dioxide method are to be found in the review of Caldwell (2). Ad-

ditional recent experiments in which the carbon dioxide method was used for measurement of the pH of mammalian muscle are those of Tobin (18) with cats and Nichols (22) with rats. Reports of the relationship between carbon dioxide tension of blood and intracellular pH of mammalian muscle as measured by the carbon dioxide method (6, 19, 22) are in general accord with the results of our present experiments with the DMO method. While our experiments showed some small effect of blood pH on muscle pH aside from changes of pCO_2 and those of Wallace and Hastings (19) and of Tobin (18) with the carbon dioxide method failed to do so, the results from the use of both methods are all indicative that muscle pH is not influenced to any great extent by blood pH *per se*. Although there has been no direct, simultaneous comparison of the two methods, it may be concluded that there is no conspicuous discrepancy between the results of the carbon dioxide method and those of the DMO method either as to the value for intracellular pH of normal muscle or as to the manner in which this value is affected by changes of blood chemistry.

The principal critic of the carbon dioxide method has been Conway (1, 3). Conway and Fearon (3) found that in alkaline extracts of muscle a large part of the acid-labile carbon dioxide is not precipitable by barium chloride. From their experiments they concluded that much of the acid-labile carbon dioxide of muscle is not bicarbonate. If the barium-soluble fraction is considered not to have come from bicarbonate in muscle, a much lower value for the calculated intracellular pH is obtained. The value so calculated is near 6.0, approximately that to be expected if the hydrogen ion ratio across the cellular membrane were governed by a Donnan equilibrium. This is also approximately the pH value estimated by Conway (1) from a balance of the ionic charges in muscle fibers. Conway concedes that the data from which this latter calculation must be made can at present only be considered approximate.

Hill (23) has argued that a Donnan equilibrium for hydrogen ions in muscle cannot be reconciled with his experiments with frog muscle. Muscles remained excitable in media of pH values such that the operation of a Donnan equilibrium would have resulted in intracellular pH values too low to be compatible with contractility. Conway (1) has sought to refute Hill's argument in terms of the long time required for attainment of equilibrium.

In the determination by Danielson, Chu and Hastings (8) of the pK′ of carbonic acid in muscle brei it was found that the calculated pK′ values did not differ significantly among themselves in experiments in which the protein concentration and the pH varied over considerable ranges. These results could not have been obtained if any large part of the acid-labile carbon dioxide had been derived from a source other than bicarbonate. However, in these experiments the ground muscle had been diluted, sodium hydroxide and sodium bicarbonate had been added, and equilibration had been carried out with various tensions of carbon dioxide. Of the total carbon dioxide released by acid from the final preparations, only a small part had originally been in the muscle. An interfering substance that might constitute an important proportion of the carbon dioxide of the muscle itself might be in such small proportion in the final preparation that the effect of its presence might not exceed the experimental error. The experiments of Danielson and co-workers (8) are not, accordingly, conclusive evidence of the absence of a significant amount of acid-labile carbon dioxide other than bicarbonate in muscle.

There is no question of the chemical identity of the substance determined analytically in the DMO method. The fact that pH values calculated from the distribution of DMO and of carbon dioxide are in the same range supports confidence that these values are correct rather than the much lower values advocated by Conway. Whatever the chemical nature may be of the barium-soluble material in the alkaline extracts of Conway and Fearon, it appears likely that essentially all of the acid-labile carbon dioxide in the muscle cell itself is bicarbonate and that the pH values calculated on this assumption are correct.

The greater the number of indicator compounds found to give concordant results, the greater the confidence that can be placed in the values of intracellular pH calculated from the distribution of acids and bases. If unrecognized factors influence the distribution of one compound so as to invalidate the calculation of pH, it is unlikely that the same factors will be in operation with a compound of entirely different chemical structure. Studies of a number of indicator compounds of

diverse structures would therefore be of value. Bases would be of particular interest because they could furnish a more sensitive indication of pH than acids when used for tissues with intracellular pH values below the plasma pH. Comparison of bases with acids would also be of interest because the errors arising from differences in pK′ values between intracellular and extracellular water due to differences in ionic strength would be in opposite direction for acids and bases. The only base the distribution of which has hitherto been used for calculation of intracellular pH is ammonia, which has the serious drawbacks of high toxicity and difficulty of analytical determination. The special requirements for a compound to be used as an indicator of intracellular pH exclude the great majority of known acids and bases, but a systematic search might disclose other appropriate compounds.

DMO has advantages over carbon dioxide for the measurement of intracellular pH, and at the present time it appears to have almost ideal properties for this purpose. Although this initial study has been limited to muscle, there is no reason why the method could not be applied to any tissue in which the determination of extracellular water is feasible. The analytical method described here for DMO in muscle has been shown to be suitable also for liver and brain. Since DMO is a foreign compound that is not attacked metabolically, an application is possible that would not be possible with carbon dioxide, *viz.*, the determination of an overall value for the intracellular pH of the tissues of the body as a whole. This could be carried out in human subjects since all that would be entailed would be the determination of DMO in plasma and urine after administration of a measured dose, together with simultaneous determinations of total body water and extracellular body water by means of other appropriate substances. The fact that DMO is not metabolized would also make possible the use of the isotopically labeled compound to study gradients of pH within small regions of a tissue or even within individual cells.

SUMMARY

The theory of estimation of intracellular pH by means of measurement of the distribution of weak organic acids and bases is discussed. 5,5-Dimethyl-2,4-oxazolidinedione (DMO) is shown to have properties almost ideal for this purpose.

The distribution of DMO has been used to measure the intracellular pH of skeletal muscle of the dog under various conditions. The intracellular pH of normal, resting muscle averaged 7.04. The greatest changes of pH of muscle are those produced by raising the carbon dioxide tension of blood. The pH of muscle is lowered by a high tension of carbon dioxide whether or not the blood pH is lowered. Metabolic acidosis has less effect in lowering muscle pH than does high tension of carbon dioxide. Respiratory and metabolic alkalosis raise muscle pH only to a small extent.

REFERENCES

1. Conway, E. J. Nature and significance of concentration relations of potassium and sodium ions in skeletal muscle. Physiol. Rev. 1957, **37**, 84.
2. Caldwell, P. C. Intracellular pH *in* Int. Rev. Cytol., G. H. Bourne and J. F. Danielli, Eds. New York, Academic Press Inc., 1956, vol. 5, p. 229.
3. Conway, E. J., and Fearon, P. J. The acid-labile CO_2 in mammalian muscle and the pH of the muscle fibre. J. Physiol. 1944, **103**, 274.
4. Butler, T. C., Mahaffee, D., and Mahaffee, C. Metabolic demethylation of 3,5,5-trimethyl-2,4-oxazolidinedione (trimethadione, Tridione). Proc. Soc. exp. Biol. (N. Y.) 1952, **81**, 450.
5. Manery, J. F. Water and electrolyte metabolism. Physiol. Rev. 1954, **34**, 334.
6. Irving, L., Foster, H. C., and Ferguson, J. K. W. The carbon dioxide dissociation curve of living mammalian muscle. J. biol. Chem. 1932, **95**, 95.
7. Conway, E. J., and Downey, M. pH values of the yeast cell. Biochem. J. 1950, **47**, 355.
8. Danielson, I. S., Chu, H. I., and Hastings, A. B. The pK_1' of carbonic acid in concentrated protein solutions and muscle. J. biol. Chem. 1939, **131**, 243.
9. Deane, N., and Smith, H. W. The apparent first dissociation constant, pK_1', of carbonic acid in the human erythrocyte. J. biol. Chem. 1957, **227**, 101.
10. Waddell, W. J., and Butler, T. C. Renal excretion of 5,5-dimethyl-2,4-oxazolidinedione (product of demethylation of trimethadione). Proc. Soc. exp. Biol. (N. Y.) 1957, **96**, 563.
11. Stoughton, R. W. 5,5-Dialkyl-2,4-oxazolidinediones. J. Amer. chem. Soc. 1941, **63**, 2376.
12. Butler, T. C. Quantitative studies of the demethylation of trimethadione (Tridione). J. Pharmacol. exp. Ther. 1953, **108**, 11.
13. Waddell, W. J., and Butler, T. C. The distribution and excretion of phenobarbital. J. clin. Invest. 1957, **36**, 1217.

14. Van Slyke, D. D., and Hiller, A. Application of Sendroy's iodometric chloride titration to protein-containing fluids. J. biol. Chem. 1947, **167**, 107.
15. Van Slyke, D. D., and Neill, J. M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. J. biol. Chem. 1924, **61**, 523.
16. Swan, R. C., and Pitts, R. F. Neutralization of infused acid by nephrectomized dogs. J. clin. Invest. 1955, **34**, 205.
17. Schwartz, W. B., Ørning, K. J., and Porter, R. The internal distribution of hydrogen ions with varying degrees of metabolic acidosis. J. clin. Invest. 1957, **36**, 373.
18. Tobin, R. B. Plasma, extracellular and muscle electrolyte responses to acute metabolic acidosis. Amer. J. Physiol. 1956, **186**, 131.
19. Wallace, W. M., and Hastings, A. B. The distribution of the bicarbonate ion in mammalian muscle. J. biol. Chem. 1942, **144**, 637.
20. Singer, R. B., Clark, J. K., Barker, E. S., Crosley, A. P., Jr., and Elkinton, J. R. The acute effects in man of rapid intravenous infusion of hypertonic sodium bicarbonate solution. I. Changes in acid-base balance and distribution of the excess buffer base. Medicine (Baltimore) 1955, **34**, 51.
21. Swan, R. C., Axelrod, D. R., Seip, M., and Pitts, R. F. Distribution of sodium bicarbonate infused into nephrectomized dogs. J. clin. Invest. 1955, **34**, 1795.
22. Nichols, G., Jr. Serial changes in tissue carbon dioxide content during acute respiratory acidosis. J. clin. Invest. 1958, **37**, 1111.
23. Hill, A. V. The influence of the external medium on the internal pH of muscle. Proc. roy. Soc. B 1955, **144**, 1.

SPECIAL NOTICE TO SUBSCRIBERS

Post Offices will no longer forward the Journal when you move.

Please notify The Journal of Clinical Investigation, Business Office, 333 Cedar Street, New Haven 11, Conn., at once when you have a change of address, and do not omit the zone number if there is one.

EFFECTS OF REDUCED GLOMERULAR FILTRATION ON URINE CONCENTRATION IN THE PRESENCE OF ANTIDIURETIC HORMONE *

By NORMAN G. LEVINSKY, DOUGLAS G. DAVIDSON AND ROBERT W. BERLINER
WITH THE TECHNICAL ASSISTANCE OF AGNES S. PRESTON

(From the Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute, Bethesda, Md.)

(Submitted for publication October 10, 1958; accepted January 2, 1959)

In the presence of antidiuretic hormone (ADH), the concentration of the urine is usually related inversely to the excreted osmotic load. When solute excretion is increased by infusion of relatively nonreabsorbable solute, urine concentration falls toward plasma concentration. Maximum urine concentrations are achieved only when solute excretion is very low. In the dog, the highest urine osmolalities, in the range of 1,800 to 2,200 mOsm. per Kg. H_2O, occur when solute excretion is about 200 to 300 μOsm. per minute. If urine concentration and solute excretion are inversely related even at the lowest solute loads, one would expect an increase in urine concentration when spontaneously low osmotic loads are decreased further by lowering the glomerular filtration rate (GFR). On the other hand, if the highest urine concentrations seen under spontaneous conditions represent the maximum osmolality which the kidney can achieve (1), further reductions in solute excretion would not be expected to result in increased urine concentration. Under certain circumstances in the present studies, maximum spontaneous urine concentrations did increase when the GFR was reduced, in conformity with the first view described.

However, neither of these alternatives explains the findings of Leaf, Kerr, Wrong and Chatillon (2), confirmed in this paper, that urine osmolality often falls when the GFR is reduced in the presence of ADH. Recently, it has been postulated (3, 4) that urine is made hypertonic to plasma by equilibration with hypertonic interstitial fluid in the medulla of the kidney. This paper presents data consistent with the view that the fall in urine concentration when GFR is reduced is due to decreased delivery to the medulla of the sodium salts and urea necessary to maintain hypertonicity of the interstitial fluid. In particular, the data support the hypothesis that urea plays a unique role in the concentrating mechanism.

METHODS

Studies were performed on female mongrel dogs weighing 16 to 22 Kg. The dogs were prepared for repeated use by positioning an inflatable silicone rubber cuff surgically around the right renal artery to permit acute, graded, reversible reduction of the GFR in the right kidney. Urine was collected separately from each kidney by means of a bladder-splitting operation. This surgical preparation [1] has been described in detail elsewhere (5).

The dogs were unanesthetized and trained to stand quietly, partially supported by slings. Water and food were withheld during the 24 to 48 hours prior to each experiment. In addition, 50 or 100 mU per Kg. per hour of vasopressin (Pitressin®) was given in the intravenous infusions throughout each experiment. The pH of the infusions was adjusted to 5.2 to 5.6 with acetic acid to assure stability of the vasopressin during the course of the experiment. For experiments in which minimal solute excretion was desired, the vasopressin, inulin and *p*-aminohippurate (PAH) were dissolved in water or 0.45 per cent saline and infused at rates of less than 0.5 ml. per minute by means of a constant infusion pump. For experiments involving osmotic diuresis, the vasopressin, inulin and PAH were added to hypertonic (6 to 10 per cent) mannitol in water or urea in 0.9 per cent saline, and the infusions were administered at appropriate rates (usually 4 to 6 ml. per minute). Collection periods were begun by attaching a short length of rubber tubing to the funnel in each half-bladder and stopped by removing the tubing. Therefore, a short interval elapsed between collection periods. The urine was allowed to drain freely into graduates during each period.

In experiments in which kidney tissue was analyzed, several consecutive collection periods with stable urine flow and osmolality were obtained immediately before the kidneys were removed. The dog was killed with

* This paper was presented in summary form at the Annual Meeting of the American Society for Clinical Investigation, May 5, 1958.

[1] We are indebted to Dr. Clarence Weldon and Dr. John Holman for the surgical preparations.

pentobarbital given rapidly intravenously in a dose sufficient to cause circulation to stop within a few seconds. The kidneys were removed from the animal within two minutes after death. The brown cap of cortex and the reddish outer medulla (strictly, the outer stripe of the outer medulla) were identified by gross anatomic characteristics and random pieces were taken for analysis. Serial sections of the (inner) medulla, from the papillary crescent toward the cortex, were cut freehand with a large, flat blade. The plane of the sections was perpendicular to the axis of the medulla. The first section, about 3 mm. thick, including the crescent and trimmed at the ends of the area cribrosa, was arbitrarily designated the papilla for purposes of these analyses. The next serial section, 3 mm. in thickness, was discarded. The section thereafter, also about 3 mm. thick, trimmed just within the red stripe of outer medulla, was called the inner medulla.[2] The pieces of inner medulla and papilla used weighed approximately 200 to 250 mg. Each tissue was weighed and homogenized in 10 or 15 ml. of demineralized water. The homogenate was divided immediately into two parts. The portion for urea analysis was centrifuged and aliquots of the supernatant were taken for analysis. The portion for electrolyte analysis was heated at 100° C. for one hour before centrifugation. Aliquots of the supernatant were then taken for sodium, potassium and chloride analysis. Other pieces of tissue from each area of the kidney were dried to constant weight under vacuum to permit calculation of tissue water as per cent of wet weight. Urea analysis was performed in duplicate by the micro-diffusion method of Conway (7); an ammonia blank was determined for each sample. Other methods of analysis used in this laboratory have been listed elsewhere (5).

RESULTS

Before each experiment, two or three preliminary collection periods were obtained to ascertain that urine osmolalities and filtration rates were comparable on the two sides. Dogs were not used if the two kidneys differed in these respects by more than about 10 per cent. The surgical preparation permitted the use of the left kidney as a simultaneous control for the right during periods in which the filtration rate of the right kidney was reduced. Spontaneous variations in urine concentration and GFR were frequent in both kidneys during preliminary periods and in the control kidney during periods when the cuff was inflated. No attempt has been made to account for these variations. The assumption is made that spontaneous changes affect both kidneys equally and that therefore the best control for the right kidney when its filtration rate is reduced is the simultaneous behavior of the unoperated left kidney. For this reason, data in summary charts are presented in terms of the ratios of the urine osmolalities or filtration rates of the two kidneys.

Data from 17 experiments performed on 12 different dogs during low solute excretion are presented in this fashion by the solid dots in Figure 1. When the GFR of the right kidney was reduced below that of the control kidney by 30 per cent or less, the urine from the right kidney usually became more concentrated than that from the control. Regularly with decreases of more than 30 per cent, and sometimes with lesser decreases in filtration rate, urine from the right kidney became much less concentrated than the urine from the control kidney. The osmolality of urine from the

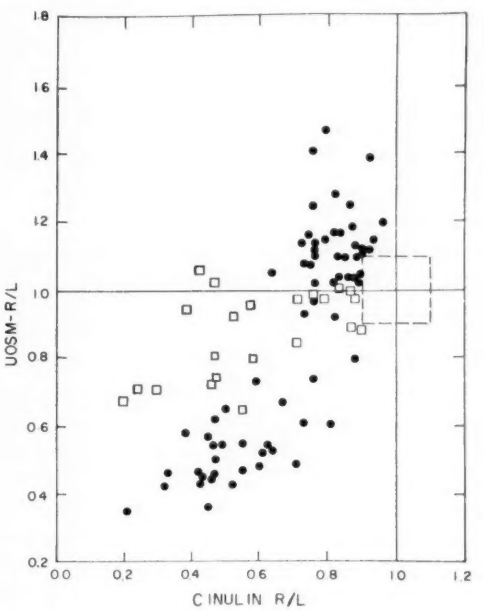


FIG. 1. CHANGES IN URINE CONCENTRATION WHEN THE GFR IS REDUCED AT LOW SOLUTE EXCRETION IN DOGS ON A REGULAR DIET (●) AND IN DOGS ON A LOW PROTEIN DIET (□)

The dashed lines circumscribe the area in which data from all the preliminary periods fall.

[2] The exact concentration gradients of urea and sodium in the medulla are not delineated well by this method, since relatively thick, nonconsecutive slices of tissue are used. However, the method is suitable and convenient for comparing concentrations in different kidneys. The use of thin, consecutive slices, as described by Ullrich and Jarausch (6), gives more precise information about concentration patterns in the medulla.

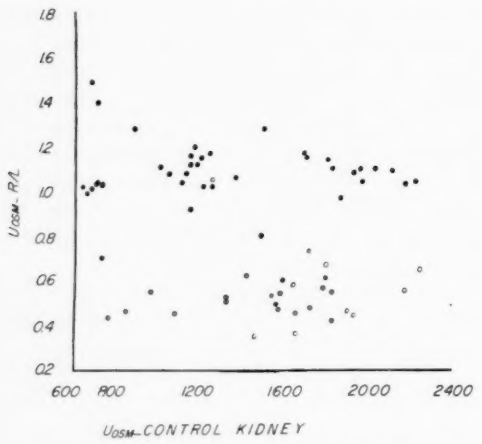


FIG. 2. CHANGES IN URINE CONCENTRATION AS A FUNCTION OF THE OSMOLALITY OF THE URINE FROM THE CONTROL KIDNEY, DURING REDUCTION OF THE GFR AT LOW SOLUTE EXCRETION IN DOGS ON A REGULAR DIET

The clearance periods represented are the same as those shown by solid dots in Figure 1. The open circles in this figure represent periods in which the ratio of the filtration rates of the two kidneys was less than 0.7; the solid dots represent periods in which this ratio was 0.7 or more.

control kidney in this group of experiments varied from 650 to 2,000 mOsm. per Kg. H_2O. The results of experiments throughout this range of concentrations were comparable, as is shown in Figure 2.

The protocol of an experiment in which a small relative reduction in GFR resulted in an absolute rise in urine osmolality is presented in Table I. The preliminary periods of this experiment are an example of the spontaneous changes in urine osmolality frequently seen in dehydrated dogs during these studies. The variations in urine concentration of both kidneys during these periods and in the control kidney during the experimental periods cannot be related to changes in solute excretion and no adequate explanation for them can be offered. These preliminary periods are therefore not to be construed as "control" periods, but do indicate that the function of the two kidneys was nearly equal. When the cuff around the right renal artery was inflated, a reduction of about 15 per cent in the GFR of the right kidney relative to the left was obtained. The osmolality of the urine from the control kidney was in the range of the highest spontaneous concentrations found in dehydrated dogs. Nevertheless, the small reduction in GFR of the right kidney resulted in a further increase in urine concentration. Immediately after the last period, the dog was killed and the kidneys removed for analysis. Urea, sodium, potassium and chloride concentrations were comparable in the two kidneys.

The protocol of a similar experiment is presented in Table II. During the preliminary periods, the function of the two kidneys was nearly equal. The cuff around the right renal artery was then inflated, and after a considerable period of adjustments, a fairly stable reduction in the GFR of the right kidney was obtained. Even at the very high urine osmolalities in this experiment, a reduction of 10 to 25 per cent in GFR resulted in a concentration difference of some 75 to 250 mOsm. per Kg. H_2O between the urine from the two kidneys. Urea concentration was somewhat greater in the papilla from the right kidney, which had excreted the more concentrated urine, than in the control papilla. Sodium, potassium and chloride concentrations were comparable in the two kidneys. Kidney analyses comparable to those obtained in these experiments were found in two dogs in which imperfect placement of the renal artery cuff resulted in a small chronic reduction in GFR and increased urine concentration in the right kidney.

In Table III, data are given from an experiment in which reduction of the GFR resulted in a decrease in urine concentration. After the cuff around the right renal artery was inflated, a stable reduction of about 50 per cent was obtained in the GFR of the right kidney as compared to the control kidney. Urine concentration from the right kidney fell to less than half the value for the left. A large part of the fall in urine osmolality was due to the fall in urine urea concentration. Analysis of kidney tissue showed marked reduction in urea concentration and small decreases in sodium and chloride concentration in the right papilla as compared to the left. Qualitatively similar data were obtained in three other experiments and from three dogs in which an imperfectly positioned artery cuff chronically reduced urine concentration and GFR in the right kidney. In some of these experiments, however, especially those in which the decrease in GFR was more than 50

TABLE I

Reduction of glomerular filtration rate with increase in urine osmolality

Time	Urine flow R	Urine flow L	PAH clearance R	PAH clearance L	Inulin clearance R	Inulin clearance L	Urine osmolality R	Urine osmolality L	Urine urea R	Urine urea L	Urine sodium R	Urine sodium L	Urine potassium R	Urine potassium L
min.	*ml./min.*		*ml./min.*		*ml./min.*		*μOsm./Gm. H_2O*		*μM/ml.*		*μEq./ml.*		*μEq./ml.*	
0	Start infusion: 10 mg./min. inulin, 5 mg./min. PAH in water at 0.2 ml./min.													
63–79	0.17	0.16	204	194	67	64	1,908	1,908						
80–97	0.15	0.15	147	141	50	50	1,420	1,419	681	680	37	34	231	228
101–114	0.23	0.22	177	172	56	54	1,227	1,235						
118–132	0.20	0.19	167	153	51	48	1,379	1,382	584	586	121	123	213	213
178	Cuff on right renal artery inflated													
234–250	0.13	0.16	134	165	60	70	2,239	2,173						
252–270	0.13	0.15	140	150	61	69	2,317	2,240	1,210	1,152	123	130	371	360
273–290	0.13	0.14	141	147	64	65	2,303	2,114						
293–309	0.10	0.13	112	125	51	57	2,244	2,032	1,094	996	254	275	287	252
215–231	0.10	0.14	116	145	51	61	2,149	1,962	1,077	984	246	276	247	214

Tissue analysis*	Urea R	Urea L	Sodium R	Sodium L	Potassium R	Potassium L	Chloride R	Chloride L	Water R	Water L
	μM/Gm. H_2O		*μEq./Gm. H_2O*		*μEq./Gm. H_2O*		*μEq./Gm. H_2O*		*% wet wt.*	
Cortex	33	26	102	94	69	75	84	72	79.2	77.8
Outer medulla			143	148	67	67	117	116	80.1	79.0
Inner medulla	539	533	262	252	42	54	235	198	83.5	80.4

* Values for papilla invalidated by technical error.

per cent, the sodium concentration of the right papilla was reduced more markedly (20 to 40 per cent less than the left papilla) than in the experiment shown in Table III. In all these cases, the reduction in tissue urea concentration was proportionately much greater than the decrease in tissue sodium concentration.

From these experiments it seemed probable that decreases in urine concentration during reduction in GFR were related to decreased delivery of urea and sodium to the medulla. During osmotic diuresis due to infusion of urea, relatively large amounts of urea and sodium appear in the urine, even when the GFR is reduced. It therefore was

TABLE II

Reduction of glomerular filtration rate with increase in urine osmolality

Time	Urine flow R	Urine flow L	PAH clearance R	PAH clearance L	Inulin clearance R	Inulin clearance L	Urine osmolality R	Urine osmolality L	Urine urea R	Urine urea L	Urine sodium R	Urine sodium L	Urine potassium R	Urine potassium L
min.	*ml./min.*		*ml./min.*		*ml./min.*		*μOsm./Gm. H_2O*		*μM/ml.*		*μEq./ml.*		*μEq./ml.*	
0	Start infusion: 10 mg./min. inulin, 5 mg./min. PAH in water at 0.2 ml./min.													
52–70	0.12	0.12	150	141	48	45	2,174	2,116	1,100	1,033	176	191	197	186
73–93	0.11	0.11	153	146	47	46	2,098	2,116	1,010	1,021	173	167	156	152
98–122	0.088	0.092	147	147	39	39	1,887	1,898	896	872	137	125	159	159
130	Cuff on right renal artery inflated													
368–390	0 083	0.092	113	120	39	42	2,086	1,932	1,098	969	214	244	194	160
393–416	0.074	0.096	98	121	39	47	2,044	1,971	946	979	222	257	173	145
418–441	0.063	0.091	82	108	34	44	2,012	1,827	1,034	947	233	251	158	124
444–458	0.061	0.088	77	101	33	43	2,055	1,808	990	952	223	242	132	105

Tissue analysis	Urea R	Urea L	Sodium R	Sodium L	Potassium R	Potassium L	Chloride R	Chloride L	Water R	Water L
	μM/Gm. H_2O		*μEq./Gm. H_2O*		*μEq./Gm. H_2O*		*μEq./Gm. H_2O*		*% wet wt.*	
Cortex	21	25	104	102	79	74	92	88	81.0	81.3
Outer medulla			154	146	78	73	142	130	83.5	79.8
Inner medulla	356	322	206	240	40	58	255	234	85.6	81.3
Papilla	881	740	337	328	50	48	320	300	81.4	81.7

TABLE III

Reduction of glomerular filtration rate with decrease in urine osmolality

Time	Urine flow R	Urine flow L	PAH clearance R	PAH clearance L	Inulin clearance R	Inulin clearance L	Urine osmolality R	Urine osmolality L	Urine urea R	Urine urea L	Urine sodium R	Urine sodium L	Urine potassium R	Urine potassium L
min.	*ml./min.*		*ml./min.*		*ml./min.*		*μOsm./Gm. H_2O*		*μM/ml.*		*μEq./ml.*		*μEq./ml.*	
0	Start infusion: 8.5 mg./min. inulin, 4.75 mg./min. PAH in water at 0.21 ml./min.													
58–87	0.07	0.06	89	96	25	27	1,700	1,814						
91–112	0.11	0.10	110	126	30	36	1,461	1,506	814	856	29	24	167	181
115	Cuff on right renal artery inflated													
303–313	0.16	0.19	78	120	25	52	746	1,655						
314–324	0.13	0.23	69	138	24	54	882	1,634	200	671	60	268	272	188
326–340	0.11	0.22	79	140	27	49	743	1,589	216	598		310		185

Tissue analysis	Urea R	Urea L	Sodium R	Sodium L	Potassium R	Potassium L	Chloride R	Chloride L	Water R	Water L
	μM/Gm. H_2O		*μEq./Gm. H_2O*		*μEq./Gm. H_2O*		*μEq./Gm. H_2O*		*% wet wt.*	
Cortex	16	21	92	93	82	91	61	70	78.5	76.7
Outer medulla			129	158	72	81	91	125	80.4	76.7
Inner medulla	131	182	221	264	46	51	160	192	84.9	81.3
Papilla	204	491	268	312	50	53	178	301	83.1	78.7

a reasonable assumption that delivery of urea and sodium to the medulla is adequate during urea diuresis even when filtration is restricted. During mannitol diuresis, urinary sodium excretion is increased and the concentration of urea in the urine is so low as to contribute an insignificant fraction of total urinary osmolality. It might be expected, therefore, that adequate amounts of sodium would reach the medulla even when the GFR is reduced during mannitol diuresis, and that changes in urea concentration could result only in minimal changes in urine osmolality. From these considerations, there should be no fall in urine osmolality when the GFR is reduced during urea or mannitol diuresis.

The results of five experiments in five different dogs during mannitol diuresis and three experiments in two different dogs during urea diuresis are plotted in Figure 3. Osmolality of the urine from the control kidney was from 350 to 550 mOsm. per Kg. H_2O in the mannitol experiments, and from 450 to 650 when urea was given. Even

TABLE IV

Reduction of glomerular filtration rate during urea diuresis

Time	Urine flow R	Urine flow L	PAH clearance R	PAH clearance L	Inulin clearance R	Inulin clearance L	Urine osmolality R	Urine osmolality L	Urine urea R	Urine urea L	Urine sodium R	Urine sodium L	Urine potassium R	Urine potassium L
min.	*ml./min.*		*ml./min.*		*ml./min.*		*μOsm./Gm. H_2O*		*μM/ml.*		*μEq./ml.*		*μEq./ml.*	
0	Start infusion: 9.6 mg./min. inulin, 4.8 mg./min. PAH, 100 mU/Kg./hr. vasopressin, 2,650 μM/min. urea, in 0.9% saline at 5.3 ml./min.													
55–61	1.7	1.8	187	127	70	71	775	764						
62–67	1.7	1.8	200	176	73	75	769	743						
69–74	1.8	2.0	124		71	72	716	694	418	382	106	106	54	48
75	Cuff on right renal artery inflated													
249–255	1.1	5.0	265	286	49	67	975	605						
257–262	1.2	5.2	270	289	45	55	924	612						
263–267	1.2	5.3	280		49	71	972	612	858	332	22	130	32	23
268	Cuff pressure increased													
274–279	0.52	5.2	174		26	62	1,040	618						
281–286	0.73	5.5	222	314	39	66	1,016	614						
288–293	0.81	5.5	204	414	43	61	985	618	890	334	6	136	26	26

TABLE V

Reduction of glomerular filtration rate during mannitol diuresis

Time	Urine flow		PAH clearance		Inulin clearance		Urine osmolality		Urine urea		Urine sodium		Urine potassium	
	R	L	R	L	R	L	R	L	R	L	R	L	R	L
min.	*ml./min.*		*ml./min.*		*ml./min.*		*μOsm./Gm. H_2O*		*μM/ml.*		*μEq./ml.*		*μEq./ml.*	
0	Start infusion: 11 mg./min. inulin, 5.5 mg./min. PAH, 110 μEq./min. sodium chloride, 100 mU/Kg./hr. vasopressin in 6% mannitol at 5.5 ml./min.													
45–53	3.2	3.2			30	32	459	462						
55–62	3.5	3.4	272	270	29	31	421	440			97	108	21	22
64–71	3.4	3.5	240	246	25	29	397	401						
75	Cuff on right renal artery inflated													
270–276	0.78	4.3	84	169	7	27	437	392						
280–285	1.2	4.4	117	145	11	27	435	392	32	26	12	40	12	13
286–291	1.3	4.3	104	121	11	27	438	393						
295	Cuff pressure adjusted													
320–324	1.5	4.4	128	160	12	25	432	393						
324–329	1.8	4.4	100	110	15	25	446	397						
330–335	1.8	4.4	90	113	15	24	450	396	29	26	5	36	17	15

when the GFR of the right kidney was reduced as much as 90 per cent during urea diuresis and 60 per cent during mannitol diuresis, urine from the right kidney regularly became more concentrated than that from the control kidney. When urea was administered, the increases in urine concentration were often very striking and regularly greater than those occurring during mannitol diuresis. An example of an experiment with urea infusion is given in Table IV and a representative experiment in which mannitol was given is shown in Table V. In the experiment shown in Table IV, the preliminary periods show approximate equality of kidney function. During the first group of three periods after the artery cuff was inflated, the GFR of the right kidney was reduced some 20 to 30 per cent. When cuff pressure was increased, GFR was decreased 30 to 60 per cent. In both groups of periods, urine from the right kidney was 50 to 60 per cent more concentrated than that from the left. The concentration difference in urea between the urines from the two kidneys was greater than the difference in urine osmolality. In the experiment shown in Table V, reductions in the GFR of the right kidney of from 40 to 75 per cent resulted in increases of 10 to 15 per cent in the concentration of the urine from the right kidney as compared to the control. Urine urea concentrations accounted for only some 5 to 7 per cent of the total osmolality of the urine from either kidney. These results during osmotic diuresis, in which urine concentration regularly rose at all degrees of reduction of the GFR, are to be contrasted with those during low solute excretion, in which urine concentration always fell when GFR was decreased more than 30 per cent (Figure 1).

In five experiments on four dogs, the urea concentration in urine from the control kidney during maximum dehydration was decreased to between 40 and 150 μM per ml. by keeping the dogs on a low protein diet for one to two weeks. These dogs were then studied without solute diuresis, in a manner analogous to the dehydrated dogs on regular diets. As is shown by the open squares in Figure 1, reductions of filtration rate resulted in smaller falls in urine concentration than did comparable reductions in dogs on regular diets. The osmolality of the urines from the control kidney varied from 400 to 900 mOsm. per Kg. H_2O in the low protein experiments. In these experiments, control urea concentrations were already so low that even complete disappearance of urea from the urine during reductions in GFR could effect little change in urine osmolality. In this respect, the experiments are analogous to those during mannitol diuresis. However, no diuretic was present in these experiments to decrease proximal sodium reabsorption, and delivery of sodium to the medulla was therefore probably restricted.

Animals were not sacrificed to obtain kidney analyses in the experiments involving osmotic diu-

TABLE VI

Kidney analysis during low protein diet, mannitol diuresis and urea diuresis

	Urea $\mu M/Gm.\ H_2O$		
	Low protein diet, for 8 days*	Mannitol diuresis†	Urea diuresis‡
Cortex	14	5	34
Outer medulla		5	56
Inner medulla	52	11	104
Papilla	52	18	169
Urine	54	25	235

* Dog on low protein diet: urine flow, 0.1 ml./min.; osmolality, 506 mOsm. per Kg. H_2O.

† Dog during mannitol diuresis: urine flow, 4.1; osmolality, 455.

‡ Dog during urea diuresis: urine flow, 2.5; osmolality, 565.

resis or low protein diet. Such analyses have been performed in a number of unoperated dogs during urea or mannitol diuresis and on low protein diets comparable to those in the experiments described in this paper. In Table VI a representative urea analysis for each category of experiment is shown. As has been true in a wide variety of states of diuresis studied in this laboratory (except when urine flow is 0.1 ml. per minute or less), the concentration of urea in the total tissue water of the papilla (including that in thinner sections taken closer to the tip of the papilla) is some 5 to 40 per cent less than the urine urea concentration.[3] Thus, tissue urea concentrations are very low in mannitol diuresis and on the low protein diet, moderate in urea diuresis.

DISCUSSION

In experiments at low solute excretion, urine concentration regularly fell when the filtration rate was reduced 30 per cent or more in the presence of ADH. Similar observations were made in dehydrated dogs by Leaf and co-workers (2). Raisz and Scheer (9) found that urine concentration fell in hydropenic man when solute excretion was decreased to very low levels. These observations are clearly the reverse of expectations derived from the usual inverse relation between solute excretion and urine concentration. In the present experiments, moderate decreases in effective renal blood flow (PAH clearance) accompanied the decreases in GFR. However, comparable reductions of renal blood flow occurred during experiments involving mannitol and urea diuresis, in which urine concentration always increased. In one experiment during urea diuresis, renal blood flow in the right kidney was reduced more than 90 per cent when the artery cuff was inflated, yet urine concentration became 60 per cent higher than the concentration of the urine from the left kidney. Sodium reabsorption and potassium excretion were not impaired in these experiments. These facts indicate that the decreases in urine concentration are not due to anoxia or to lack of essential substrates because of restricted blood flow. Comparable falls in urine concentration occurred when control urine concentration was anywhere in the range from 650 to 2,000 mOsm. per Kg. H_2O. At similar control urine concentrations, urine osmolality increased or fell depending on the degree of reduction of GFR and the presence or absence of an osmotic diuretic. Thus, the initial degree of urine concentration *per se* was not the critical factor in these experiments.

The data presented in this paper are all consistent with, and readily explained by, the view that the formation of hypertonic urine depends on the delivery of sodium and urea to the medulla and that these are restricted when GFR is re-

[3] Recent experiments in this laboratory (8) have shown that when hypertonic urine passes through the ureter and bladder, particularly at flows of 0.2 ml. per minute or less, osmolality decreases, largely because of diffusion outward of urea, though smaller movements of water and electrolytes along their concentration gradients also occur. Ullrich and Jarausch (6) found that the maximum urea concentration in the papilla is usually equal to that in the urine. Urine flow was presumably low in their experiments, and urine was allowed to pool in the bladder before and for some minutes after the dogs were killed. From the data obtained in this laboratory, one would estimate that urea concentrations in urine collected in this way were 10 to 40 per cent less than in the urine leaving the kidney.

Because of the changes in urine osmolality and urea concentration in the ureter and bladder, no exact significance can be assigned to the urine concentrations at low flows given in this paper. Since these changes are increased as flow is reduced, the rises in urine concentration which actually occur when GFR is decreased in experiments like that in Table II are probably about 10 per cent greater than those found. In experiments in which urine osmolality and urea concentration fell, such as that shown in Table III, the actual decreases are presumably about 20 per cent less than those observed.

duced. Wirz (3) has proposed that the urine is made hypertonic in the collecting ducts by equilibration with hypertonic medullary interstitial fluid. The available evidence supports the hypothesis that hypertonicity is established in the medullary interstitial fluid by means of active transport of sodium salts in excess of water by some part of the loop of Henle, though the exact mechanism is unknown (3, 4, 6).[4] Ullrich and Jarausch (6) have found high concentrations of sodium in the medulla, and these results are confirmed by numerous analyses in this laboratory. For example, the concentration of sodium in the total tissue water of the papilla, as shown in Table I, is more than twice that of plasma. Urine sodium concentration is usually much lower than the concentration in the total tissue water of the medulla, so that the latter value would be reduced by virtue of the urine contained in the medulla. Moreover, it is unlikely that the cells of the Henle loops and collecting ducts contain very high concentrations of sodium. The sodium concentration in medullary interstitial fluid is therefore probably much higher than the value found for total tissue water.

Urea concentrations in the medulla are also very high, but in our experience the concentration of urea in total tissue water is always 5 to 40 per cent lower than the urea concentration of the urine, whenever urine flow is 0.1 ml. per minute or more. The following hypothesis (4) accounts for the high urea concentrations in the medulla, and in addition assigns a unique role to urea in the concentrating mechanism. Even at low urine flows, some 30 to 40 per cent of the filtered urea appears in the urine (13), whereas only 0.5 to 1 per cent of the filtered water is passed in the urine. If some urea is lost during the process of making the urine hypertonic in the collecting ducts, reasonable figures for the fluid reaching the start of the ducts would be 50 per cent of the filtered urea and 2 per cent of the filtered water. Since 50 per cent of the urea is contained in 2 per cent of the filtered volume, the urea concentration of the fluid entering the collecting system would be some 25 times the blood urea concentration. If the collecting ducts are permeable to urea as well as to water, urea will diffuse out along the concentration gradient from the fluid in the collecting ducts to the medullary interstitial fluid. As water is withdrawn from the collecting ducts into the hypertonic (sodium salts) interstitial fluid, urea concentration in the fluid within the ducts continues to rise and the situation is continuously favorable for diffusion of urea into the interstitial fluid. Because of the low effective blood flow to the medulla (probably by virtue of countercurent flow in the capillaries) (4), the urea continuously fed into the interstitial space from the collecting ducts is trapped. Therefore, the total amount of urea escaping from the collecting ducts in the steady state need not be large to maintain a high concentration in the interstitial fluid. In fact, this concentration would seem to be in the range of 60 to 90 per cent of the urine urea concentration. The urine osmolality due to this fraction of urine urea is balanced osmotically in the interstitial fluid by urea itself. Hence the active mechanism for sodium in the loops of Henle need only create hypertonicity of sodium salts sufficient to balance the osmotic effects of the rest of the urea plus that due to other urine solutes. It is emphasized that all movements of urea in this proposed mechanism are passive. By contributing a fraction of the urea with which it entered, the urine in the collecting ducts can maintain a high urea concentration in the interstitium and this can balance the osmotic effects of a large fraction of the urinary urea. The position of urea in the concentrating process is thus unique in that it can add to the osmotic pressure of the urine without

[4] The recent finding of Gottschalk and Mylle (10) that fluid in the loop of the hamster has an osmolality close to that of capillary blood and urine at the same level in the papilla confirms the earlier observations of Wirz and co-workers (11) and is contrary to the hypothesis that the urine is progressively diluted as it traverses the loops (4). However, it is clear that the urine must already be dilute as it leaves the medulla in the ascending limb, not only because it has been found to be dilute in the first part of the distal convoluted tubules at the kidney surface (12) but because the balance of tonicities of the fluids entering and leaving the medulla requires this. During the formation of hypertonic urine, three isotonic fluids (proximal tubule fluid, isotonic terminal distal tubule fluid, and cortical capillary blood) enter the medulla. Since the urine leaves hypertonic and, rejecting the possibility of active solute or water transport by the capillary walls, the blood must leave this hypertonic area on the concentrated side of isotonicity, it follows that some hypotonic fluid must emerge from the area and that this hypotonic fluid must be that of the ascending limb of the loop.

being balanced by an osmotically equivalent amount of sodium salts in the medullary interstitial fluid.

The data presented in this paper are readily interpreted in terms of this hypothesis. When the GFR is substantially reduced during low solute excretion, higher urine to plasma gradients of urea and slower flows of tubular fluid result from the proportionately greater reabsorption of water. Back-diffusion (passive reabsorption) of urea in the convoluted renal tubule increases greatly, and very little urea reaches the collecting ducts. Therefore, the concentration of urea in the interstitial space of the medulla falls. In addition, the fraction of filtered sodium reabsorbed in the proximal tubule presumably increases when the GFR is reduced, and less is available for transport by the loops of Henle. Hence, interstitial fluid sodium concentration falls. Since the bulk of the hypertonicity of the interstitial fluid is due to urea and sodium salts, on both counts reduction of GFR during low solute excretion will result in decreased urine concentration.

When the dogs are on low protein diets, the contribution of urea to urine osmolality is relatively small even at control filtration rates. Little further fall in urine urea concentration can occur when GFR is reduced. Hence, falls in osmolality would be due only to decreased delivery of sodium to the medulla. Thus the fall in osmolality should be less on low protein than on regular diets, as was found (Figure 1).

The loop of Henle presumably reabsorbs only a fraction of the sodium which reaches it under ordinary circumstances (14). Therefore, in the experiment at low solute excretions in which the reduction in GFR was small, one might expect that even the somewhat restricted amount of sodium reaching the loop would still be sufficient to permit sodium transfer at roughly the previous rate. Similarly, only a modest reduction in the amount of urea reaching the collecting ducts would be expected. Since only a small amount of urea need move across the ducts to maintain a high medullary interstitial concentration, the urea concentration in the interstitium should be maintained. Thus a small reduction in GFR should not result in a fall in urine osmolality or in tissue sodium and urea concentrations, even when no osmotic diuretic is present. The data in Tables I and II and Figure 1 show this is the case.

During an osmotic diuresis, proximal sodium reabsorption is decreased (15), and it might be expected that adequate sodium would reach the loop of Henle to permit the usual levels of sodium in the medullary interstitium to be maintained, even in the face of marked reductions of GFR. During mannitol diuresis, the large volume of tubular fluid reaching the collecting ducts prevents urea concentration in the urine from achieving appreciable values. Since movement of urea from the tubular fluid into the medullary interstitial fluid is postulated to result from a passive process, the concentration of urea in the interstitium cannot be higher than urine concentration (Table VI). Urea concentrations in mannitol diuresis are already so low at normal filtration rates, that no significant drop in concentration can result from reduction of the GFR. During urea diuresis, a relatively large amount of urea at moderately high concentration should reach the collecting ducts even when GFR is reduced. Therefore, medullary urea concentration would not be expected to fall at reduced filtration rates. The results in Figure 3, which shows that urine osmolality does not fall even with marked reduction of GFR during osmotic diuresis, are in accord with these predictions.

When the solute load is reduced by lowering the filtration rate, the amount of water withdrawn in the concentrating process is decreased. This water must contain at least the sodium concentration of the plasma to leave the medulla in the capillary circulation. Therefore, the rate of loss of sodium (or any diffusible substance) from the medulla via a counter-current capillary flow will depend on the amount of water which must be carried out of the area in the blood (4). Assuming that sodium transport by the loops of Henle is approximately constant, the concentration of sodium salts in the medullary interstitial fluid will be inversely related to the amount of water entering from the collecting ducts. Since the urine finally comes into equilibrium with the interstitial fluid, the concentration of the urine will also be related inversely to the amount of water reabsorbed in the concentrating process. Therefore, reduction in this quantity when GFR and hence solute excre-

tion are decreased would be expected to cause a rise in urine osmolality. When the GFR is considerably reduced at low solute excretions, however, this effect is more than balanced by the decreased hypertonicity of the medullary fluids due to inadequate delivery of sodium and urea. Therefore, urine osmolality falls. With small reductions of GFR at low solute loads, and with all degrees of reduction studied during solute diuresis, the delivery of sodium and urea are presumed to be adequate, for the reasons already stated. Therefore, in these situations urine osmolality rises when solute excretion is reduced. In accord with the role postulated for urea in enhancing urine concentration, reduction of the GFR during urea diuresis results in a greater increase in urine osmolality than during mannitol diuresis. Results somewhat analogous to those presented in this paper have been obtained in dehydrated human subjects by Levitt, Levy and Polimeros (16). When the filtration rate was reduced moderately in normal subjects, urine osmolality fell; in uremic subjects, similar reductions in filtration rate resulted in rises in urine osmolality.

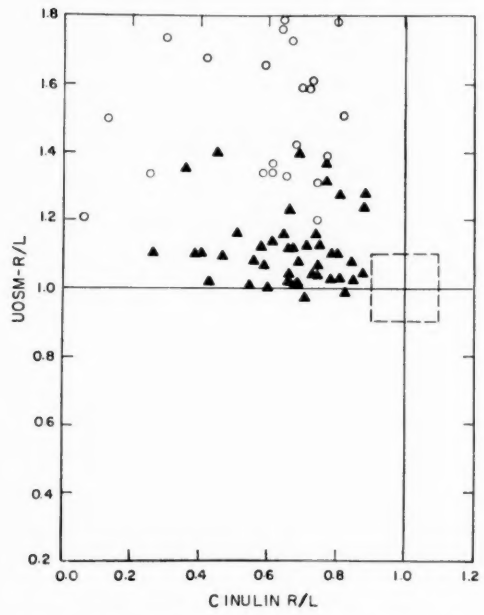


FIG. 3. CHANGES IN URINE CONCENTRATION WHEN THE GFR IS REDUCED DURING MANNITOL INFUSION (▲) AND DURING UREA INFUSION (○)

The dashed lines circumscribe the area in which data from all the preliminary periods fall.

Even at the low end of the scale of solute excretion one would expect, according to the present hypothesis, that each reduction of solute excretion would yield some further increase in urine concentration, since the amount of water moved into the medullary interstitium during the concentrating process would be progressively decreased. In the experiments reported in this paper, increases in urine concentration occurred when GFR was lowered even if control urine osmolality was in the highest range and solute excretion in the lowest seen in maximally dehydrated dogs. There was no evidence in these experiments of an approach to an "osmotic ceiling," *i.e.*, to a fixed maximum urine osmolality or maximum urine to plasma (U/P) ratio of osmolalities, such as Smith (1) has postulated. Rather, small reductions in solute excretion at any urine concentration in the range between 800 and 2,000 mOsm. per Kg. H_2O result in roughly comparable small increases in urine osmolality. However, some effect of small reductions in renal blood flow on urine concentration cannot be ruled out in these experiments.[5] An inverse relation between urine concentration and solute excretion, even at the lower solute excretions, has also been found in man (9).

SUMMARY

The effects of reductions in glomerular filtration rate (GFR) on the elaboration of hypertonic urine have been studied in dogs in which the GFR was decreased reversibly in one kidney and urine collected separately from each kidney. When the GFR of one kidney was lowered 10 to 30 per cent in dehydrated dogs infused with vasopressin, the

[5] Theoretical considerations suggest that the efficiency with which sodium salts and urea are "trapped" in the medulla is inversely related to some power (between one and two) of blood flow (4). If the medullary blood flow takes part in the slight reduction of total effective renal blood flow noted during inflation of the arterial clamp in these experiments, increased hypertonicity of the medullary fluids and consequently of the urine might occur on this basis. Since no method for measuring blood flow to the medulla is available, this possibility cannot be tested experimentally.

concentration of the urine from that kidney often became greater than that from the control kidney by amounts up to 40 per cent. Increases in urine osmolality were noted even when control urine concentration was as high as 2,000 mOsm. per Kg. H_2O. This indicates that the maximum urine concentrations found at very low osmolar excretions under ordinary conditions do not represent the maximum urine to plasma (U/P) ratio ("osmotic ceiling") which the kidney can achieve.

Regularly when the reduction of GFR was 30 per cent or more at low solute flows, and sometimes with smaller reductions in GFR, urine osmolality fell markedly. Under these conditions, analysis of renal tissue showed large decreases in urea concentration and moderate decreases in sodium chloride concentration in the medulla of the kidney whose GFR has been lowered. During urea or mannitol diuresis urine osmolality regularly increased, even when the GFR was reduced more than 60 per cent.

These data are consistent with the hypothesis that urine hypertonicity is developed by equilibration of fluid in the collecting ducts with hypertonic medullary interstitial fluid and that the medullary interstitial fluid is made hypertonic to plasma by deposition of sodium salts from the loops of Henle. Accumulation of urea in the medulla, considered to be the result of passive diffusion from urea-permeable collecting ducts, makes possible the achievement of considerably higher osmolalities than could be developed in response to the interstitial sodium salts alone.

ADDENDUM

Since submission of this paper our attention has been called to a paper (Miles, B. E., Paton, A., and DeWardener, H. E. Maximum urine concentration. Brit. med. J. 1954, **2**, 901) in which it is indicated that the capacity to form more concentrated urine when urea is a major urinary constituent may be attributable to permeability to urea of the cells responsible for the removal of water in the production of hypertonic urine. Although this proposal does not include the essential feature of trapping the urea in the peritubular area, the other similarities to the mechanism outlined in this paper are worth noting.

REFERENCES

1. Smith, H. W. Principles of Renal Physiology. New York, Oxford University Press, 1956, p. 122.
2. Leaf, A., Kerr, W. S., Jr., Wrong, O., and Chatillon, J. Y. Effect of graded compression of the renal artery on water and solute excretion. Amer. J. Physiol. 1954, **179**, 191.
3. Wirz, H. The location of antidiuretic action in the mammalian kidney *in* The Neurohypophysis, H. Heller, Ed. New York, Academic Press Inc., 1957, p. 157.
4. Berliner, R. W., Levinsky, N. G., Davidson, D. G., and Eden, M. Dilution and concentration of the urine and the action of antidiuretic hormone. Amer. J. Med. 1958, **24**, 730.
5. Berliner, R. W., and Davidson, D. G. Production of hypertonic urine in the absence of pituitary antidiuretic hormone. J. clin. Invest. 1957, **36**, 1416.
6. Ullrich, K. J., and Jarausch, K. H. Untersuchungen zum Problem der Harnkonzentrierung und Harnverdünnung. Pflüg. Arch. ges. Physiol. 1956, **262**, 537.
7. Conway, E. J. Micro-Diffusion Analysis and Volumetric Error. London, Crosby, Lockwood and Son, Ltd., 1957.
8. Levinsky, N. G., and Berliner, R. W. Amer. J. Physiol. In press.
9. Raisz, L. G., and Scheer, R. L. Impairment in renal concentrating capacity with heavy exercise. Clin. Res. Proc. 1957, **5**, 203.
10. Gottschalk, C. W., and Mylle, M. Evidence that the mammalian nephron functions as a countercurrent multiplier system. Science 1958, **128**, 594.
11. Wirz, H., Hargitay, B., and Kuhn, W. Lokalisation des Konzentrierungs-prozesses in der Niere durch direkte Kryoskopie. Helv. physiol. pharmacol. Acta 1951, **9**, 196.
12. Wirz, H. Der osmotische Druck in den corticalen Tubuli der Rattenniere. Helv. physiol. pharmacol. Acta 1956, **14**, 353.
13. Shannon, J. A. Glomerular filtration and urea excretion in relation to urine flow in the dog. Amer. J. Physiol. 1936, **117**, 206.
14. Gottschalk, C. W., and Mylle, M. Micropuncture study of osmolality of renal tubular fluid in the hydropenic rat during osmotic diuresis. Fed. Proc. 1958, **17**, 58.
15. Wesson, L. G., Jr., and Anslow, W. P., Jr. Excretion of sodium and water during osmotic diuresis in the dog. Amer. J. Physiol. 1948, **153**, 465.
16. Levitt, M. F., Levy, M. S., and Polimeros, D. Effect of reduction in filtered load on solute and water excretion in hydropenic man. J. clin. Invest. 1958, **37**, 911.

THE ROLE OF UREA IN THE URINE CONCENTRATING MECHANISM

By NORMAN G. LEVINSKY and ROBERT W. BERLINER with the technical assistance of AGNES S. PRESTON

(From the Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute, Bethesda, Md.)

(Submitted for publication October 10, 1958; accepted January 2, 1959)

Many years ago, Gamble, McKhann, Butler and Tuthill (1) described "an economy of water in renal function referable to urea." Recently, experiments in the rat (2–4) and man (5) have confirmed their suggestion that the urine volume required to excrete a given solute load is reduced when urea is a principal urinary solute. An hypothesis assigning a unique role to urea in the urine concentrating process has been presented in the preceding paper (6) and elsewhere (7). In this paper, it is demonstrated that urine concentrating ability is reduced in dogs on a low protein diet, and that this defect is reversed acutely when urea is given. A similar acute effect of urea is also shown to occur in man. The data support the hypothesis that the passive accumulation of urea in the medullary interstitial fluid accounts for its ability to enhance urine concentration.

METHODS

Studies were done on five female mongrel dogs, each 14 to 18 Kg. in weight. The dogs were fed a synthetic diet which supplied approximately 80 cal. per Kg. per day. During the control period, the diet contained about 50 Gm. per day of protein. During the "low protein" period the diet was protein-free, except that 100 mg. per day of egg albumin was added to the diet when this period was more than 10 days long.

Studies on dogs were done after 42 hours of dehydration. Twenty-four and 16 hours prior to testing, 5 units of a long-acting vasopressin preparation (Pitressin® Tannate in oil) was administered intramuscularly. A 24 hour urine collection was usually made during the last day of the dehydration period. For tests of urine concentrating ability, an infusion containing 50 mU per Kg. per hour of vasopressin, inulin and *p*-aminohippurate (PAH) in water was given at about 0.2 ml. per minute. Several collection periods were obtained to serve as a measure of maximum urine osmolality. An additional infusion of 6.5 per cent mannitol in water or 3 per cent urea in 0.9 per cent saline was then given at several rates, to permit three or four collection periods at stable urine flows to be obtained at each of several levels of solute excretion in the range from 600 to 6,000 μOsm. per minute. When the acute effects of urea on urine concentration were to be studied, only the first infusion was given. After several control periods, the urea, dissolved in 10 to 20 ml. of water, was given by stomach tube.

The dogs were standing, partially supported by slings, during clearance periods. An indwelling catheter was used, and the bladder was emptied with the aid of abdominal compression and air washouts. Inulin clearance was used as a measure of glomerular filtration rate (GFR) and PAH clearance as a measure of effective renal blood flow. The analytical methods used have been listed elsewhere (6, 8).

Kidney tissue for analysis was obtained from dogs dehydrated for 42 hours, as described above. The methods involved in tissue analysis are described in the preceding paper (6).

The acute effects of urea on urine concentration were also studied in three young male human volunteers. The urine concentration test was conducted as follows. After 21 hours of water deprivation, the subjects voided each hour for 7 to 10 hours. After several collections with stable urine osmolality had been obtained, the urea, dissolved in 30 to 50 ml. of water, was taken by mouth. This test was performed after the subjects had been on a diet containing 20 Gm. per day of protein for 6 to 10 days.

RESULTS

Effect of low protein diet on urine concentration in dogs

In Table I, the changes in urine volume and solute concentration in one dog on a low protein diet are shown. Each 24 hour collection was begun after 18 hours of dehydration; long-acting vasopressin was given twice during the collection period. After one week on the low protein diet, urine concentration had fallen to about one-half the control value. At this time, solute excretion was still approximately at control levels. Thereafter, urine concentration remained relatively stable, while solute excretion fell to about one-half the control value on the nineteenth and twenty-sixth days of the low protein diet. Thus,

TABLE I

Twenty-four hour urine solute concentrations in dog on low protein diet

	Volume	Urine osmolality	Urea conc'n	Sodium conc'n	Potassium conc'n	Plasma urea
	ml.	*mOsm./Kg. H_2O*	*mM/L.*	*mEq./L.*	*mEq./L.*	*mM/L.*
Control	170	1,609	752	226	71	5.5
7th day	365	869	111	286	85	2.6
13th day	310	851	112	283	122	2.7
19th day	183	848	55	301	124	2.3
26th day	180	893	97	97	199	2.6

urine concentration was reduced both at control and at decreased levels of solute excretion. Urine urea concentration had fallen sharply by the seventh day on low protein and remained stable thereafter. The decrease in urea concentration accounts for about 85 to 95 per cent of the fall in urine osmolality. Plasma urea fell to one-half the control value during the first week on the low protein diet and was roughly constant thereafter. The GFR was 77 ml. per minute on the control day, and 55 ml. per minute on the seventh day of low protein diet.

Figure 1 [fashioned after the comparable figure in man of Epstein, Kleeman, Pursel and Hendrikx (5)] shows the changes in maximum concentration after six to eight days on the low protein diet in the five dogs studied. Maximum urine osmolality had fallen uniformly to about one-half the control values. Thereafter, maximum urine osmolality either remained relatively constant (two dogs) or gradually decreased to slightly lower levels (three dogs) as the low protein diet was prolonged. The minimum value in the latter dogs after three to four weeks on the diet was about one-third of the control value. In all the dogs, the fall in urine urea concentration accounted for about 80 to 95 per cent of the fall in urine osmolality. The changes in plasma urea were in general comparable to those shown in Table I. In one dog, plasma urea fell sharply to less than 0.5 μM per ml. after three weeks on the low protein diet.

The glomerular filtration rate had fallen about 25 per cent by the sixth to eighth day on the low protein diet in four of the five dogs studied (77 to 48 ml. per minute; 77 to 55; 75 to 65; 90 to 71). In one dog, the filtration rate did not change during two weeks on the diet (55 ml. per minute). These figures represent the average of the three to four consecutive periods during mannitol diuresis in which the negative free-water clearance ($T^c_{H_2O}$) was highest. This value was used arbitrarily because the filtration rate was often very labile during the course of graded osmotic diuresis, so that an average of all the periods (usually 10 to 15) would not be meaningful. For example, in one dog the GFR rose from 68 to 81 ml. per minute during graded mannitol diuresis on the control diet; and fell from 67 to 58 ml. per minute during a comparable study on the low protein diet. The values during the periods of highest negative free-water clearance were 75 and 65 ml. per minute, respectively. In two dogs in which the PAH clearance was measured on the regular and low protein diets, it fell from 290 to 220, and from 250 to 170 ml. per minute, respectively.

The maximum negative free-water clearance fell sharply on the low protein diet, as is shown in

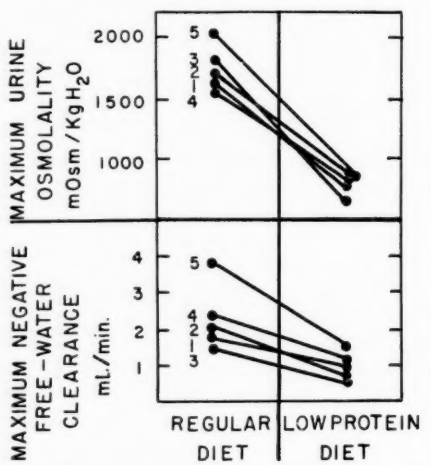


FIG. 1. MAXIMUM URINE CONCENTRATION AND MAXIMUM NEGATIVE FREE-WATER CLEARANCE IN FIVE DOGS ON REGULAR AND LOW PROTEIN DIET

Figure 1. However, certain considerations make the interpretation of changes in $T^c_{H_2O}$ in these dogs difficult. The average of the three highest consecutive $T^c_{H_2O}$ values found during graded mannitol diuresis was arbitrarily designated the "maximum" negative free-water clearance. This is not to be equated with the concept of a fixed maximum negative free-water clearance ($T^c_{mH_2O}$) approached at the highest solute excretions, as postulated by Smith(9). In these and other studies with solute diuresis, we usually did not find even an approximately constant level of $T^c_{H_2O}$. Usually, $T^c_{H_2O}$ rose up to a point as solute excretion increased, then began to fall as solute excretion was increased further. The free-water clearance often became positive at the highest levels of solute excretion (*i.e.*, the urine became hypotonic to plasma). During mannitol diuresis on the low protein diet, hypotonic urine was regularly obtained at relatively low solute excretions (C_{osm}, 10 to 16 per cent of GFR).

These facts are illustrated in Table II. In the experiment on the regular diet, $T^c_{H_2O}$ fell somewhat at the highest rates of solute excretion. In the experiment on the low protein diet, the free-water clearance became positive ($T^c_{H_2O}$ negative)

TABLE II

Solute diuresis *

Urine flow	Urine osmolality	Plasma osmolality	Cosm	$T^c_{H_2O}$	GFR	Urine urea
ml./min.	*mOsm./Kg. H_2O*	*mOsm./Kg. H_2O*	*ml./min.*	*ml./min.*	*ml./min.*	*μM/ml.*
			A. Regular diet			
0.32	1,192	306	1.25	0.9		478
0.49	1,159		1.87	1.4	57	
0.80	937		2.46	1.7	59	295
1.53	565	305	2.84	1.3		
2.95	437	305	4.13	1.3	54	72
3.50	438		5.02	1.5	62	
3.25	430	308	4.53	1.3	57	
4.63	401	306	6.06	1.4	57	
4.64	405		6.11	1.5	55	48
4.85	406	311	6.33	1.5	57	
9.42	362	320	10.70	1.2	51	
10.30	352		11.30	1.0	51	25
10.40	351		11.30	0.9	52	24
10.40	353	324	11.40	1.0	55	
			B. Low protein diet			
0.95	555	293	1.80	0.9	60	123
1.89	389		2.51	0.6	51	50
2.74	346		3.23	0.5	55	
3.22	327		3.60	0.4	57	
3.10	317	294	3.35	0.3	55	64
4.03	275	294	3.74	−0.3	53	
4.13	271		3.81	−0.3	54	62
4.16	270	294	3.82	−0.3	53	
5.63	271	301	5.17	−0.5	52	
6.16	262		5.34	−0.8	51	41
6.84	262	304	5.89	−1.0	56	
10.20	248	305	8.25	−2.0	42	16
12.70	245		10.10	−2.6	48	13
15.60	244	308	12.40	−3.3		13

* Infusions: *1*) Eight mg. per minute inulin; 4.5 mg. per minute PAH; 100 mU per Kg. per hour vasopressin in water at 0.2 ml. per minute. *2*) NaCl, 20 mM per L.; mannitol, 65 Gm. per L. in water at various rates.

In each experiment, the horizontal lines separate a group of periods at a given infusion rate. Periods were 6 to 10 minutes in length and 30 minutes was allowed before starting collections at each rate of infusion. Both experiments were performed on a single dog.

TABLE III

Acute effect of urea on urine concentration in dog on low protein diet

Time	Urine flow	Urine osmolality	Urea concentration
min.	*ml./min.*	*mOsm./Kg. H_2O*	*μM/ml.*
0	Start infusion: 50 mU/Kg./hr. vasopressin in water at 0.2 ml./min.		
39–67	0.094	1,151	284
67–99	0.087	1,139	
99–129	0.098	1,183	312
140	1.4 Gm. urea in *ca.* 10 ml. H_2O P.O.		
145–169	0.158	1,601	582
169–200	0.230	1,475	
200–232	0.217	1,551	615
232–263	0.161	1,772	
263–293	0.217	1,682	668
293–324	0.159	1,660	
324–340	0.105	1,624	678

TABLE IV

Acute effect of urea on urine concentration in man on low protein diet

Time	Urine flow	Urine osmolality	Urea concentration
min.	*ml./min.*	*mOsm./Kg. H_2O*	*μM/ml.*
0–60	0.30	753	303
60–120	0.31	751	
120–180	0.33	775	265
210	10 Gm. urea in *ca.* 30 ml. H_2O P.O.		
180–240	0.38	834	
240–300	0.55	914	489
300–360	0.63	963	
360–420	0.45	1,047	517
420–480	0.47	1,054	
480–540	0.40	1,047	601

at relatively low levels of solute excretion and the degree of hypotonicity increased as solute excretion was increased. It is emphasized that this dog had been dehydrated for 42 hours prior to each experiment, had received 10 units of long-acting vasopressin in the previous 24 hours and was receiving 100 mU per Kg. per hour of vasopressin intravenously throughout each experiment. In this dog, the GFR was relatively stable between experiments and during each experiment. The marked decrease in $T^c_{H_2O}$ at each level of solute excretion on a low protein diet as compared to the control diet evidently cannot be explained by changes in GFR in this case. At each level of solute excretion, the urea concentration of the urine is higher on the regular than on the low protein diet. This was true in the experiments in all five dogs.

Acute effect of urea on urine concentration

The acute effect of urea on maximum urine concentration was tested in seven experiments in four dogs which had been on the low protein diet for 8 to 20 days. Table III shows one such experiment. An abrupt increase in urine concentration followed the administration of a small dose of urea in spite of a concurrent rise in solute excretion. Some 80 per cent of the increase in urine osmolality can be accounted for by the increased urinary urea concentration. In four other experiments in which urine osmolality increased sharply, about 75 to 90 per cent of the increase could be accounted for by an increased urea concentration. At least one experiment in which urine osmolality rose sharply was obtained in each dog studied. In experiments in two of these same dogs, a dose of urea higher than that which produced the abrupt rise in osmolality was given. Urine concentration remained constant or fell slightly, while solute excretion increased three- to sevenfold.

In Table IV, one of three similar experiments in man is shown. As in the dog, a rise in urine concentration in spite of an increase in solute excretion follows oral administration of urea. In the three experiments in humans, rises in urine urea

TABLE V

Kidney tissue analysis in dog on low protein diet

	Urea	Sodium	Potassium	Chloride	Water
	μM/Gm. H_2O	*μEq./Gm. H_2O*	*μEq./Gm. H_2O*	*μEq./Gm. H_2O*	*% wet wt.*
Cortex	7	108	71	103	81.7
Outer medulla		197	72	180	81.0
Inner medulla	55	250	47	223	84.6
Papilla	66	282	51	236	83.4
Urine	64	95	51		

Urine flow, 0.10 ml. per minute; osmolality, 544 mOsm. per Kg. H_2O

TABLE VI

Kidney analysis in dehydrated dogs on regular diet

	Urea	Sodium	Potassium	Chloride	Water
	μM/Gm. H_2O	*μEq./Gm. H_2O*	*μEq./Gm. H_2O*	*μEq./Gm. H_2O*	*% wet wt.*
	Urine osmolality, 1,061 mOsm. per Kg. H_2O				
Cortex	9	94	76	74	78.0
Outer medulla		161	76	132	77.0
Inner medulla	167	248	38	225	83.0
Papilla	476	284	44	242	78.9
Urine	647	63	102		
	Urine osmolality, 1,794 mOsm. per Kg. H_2O				
Cortex	25	99	77	89	76.1
Outer medulla		168	71	149	77.5
Inner medulla	408	278	43	264	81.9
Papilla	780	317	45	281	76.6
Urine	1,184	73	134		

accounted for some 90 to 110 per cent of the increase in urine osmolality.

Kidney tissue analysis

The kidneys of each dog studied on the low protein diet were analyzed for sodium, potassium, chloride and urea. The dogs had been on the diet for 10 to 30 days when the analyses were made. Table V shows the complete analysis for one dog. In Figure 2, the urine urea and tissue urea and sodium concentrations of all the dogs on the low protein diet are represented by solid symbols. For purposes of comparison, complete kidney analyses in two dogs on a regular diet are presented in Table VI, and data from 19 such dogs are shown by the open symbols in Figure 2. Sodium, potassium and chloride concentrations in the kidneys of the dogs on low protein diets were within the range for these elements found in dehydrated dogs on regular diets. The average sodium concentration in the papilla of the five dogs, studied after 42 hours dehydration, was 300 ± 24 μEq. per Gm. tissue water (mean ± S.E. mean). In six dogs on the regular diet, dehy-

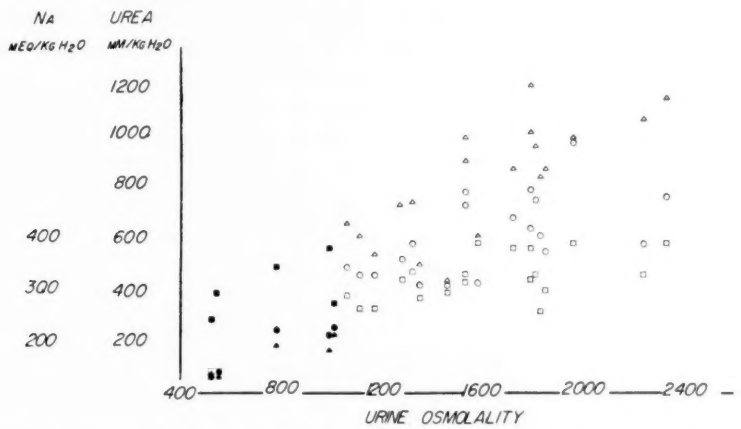


FIG. 2. KIDNEY ANALYSES IN DOGS ON REGULAR DIET (OPEN SYMBOLS) AND LOW PROTEIN DIET (SOLID SYMBOLS)

The squares represent the sodium concentration (μEq. per Gm. H_2O); the circles, urea concentration (μM per Gm. H_2O) in the tissue water of the papilla. The triangles represent urine urea concentration (μM per ml.) in the urine collected just prior to sacrifice. The abscissa shows the osmolality of this urine (mOsm. per Kg. H_2O).

drated 40 to 48 hours, papillary sodium was 300 ± 13 μEq. per Gm. tissue water.

The concentration of urea in the tissue water of the papilla was approximately equal to or slightly greater than the urinary urea concentration in each dog studied on a low protein diet. The values ranged from 50 to 250 μM per Gm. tissue water. In each case, urine flow just prior to sacrifice was 0.2 ml. per minute or less, and in three dogs it was 0.08 ml. per minute or less. In the latter dogs, the disparity between papillary and urinary urea concentration was most marked.

DISCUSSION

In experiments in which rats were fed a variety of substances separately or in mixtures, Gamble, McKhann, Butler and Tuthill (1) found that the water requirements for urinary excretion of mixed solute loads were additive except when urea was a component of the mixture. When urea was present in a mixture, urine volume was much less than the sum of the requirements for urea and the other solutes as determined separately. These results led to the conclusion that "there is an economy of water in the secretion of urine referable to urea." Although these experiments have been criticized because water intake was not controlled, more recent work indicates that the conclusions are nevertheless essentially correct. Kellogg and Koike (2) found that the negative free-water clearance at all levels of solute excretion was much higher in rats during urea infusion than during the infusion of a variety of other solutes. Radford (3) found that urine osmolality in rats is higher when urea as compared to sodium chloride is added to the diet. A marked decrease in the water requirement for excretion of a solute load when urea was added to the diet was observed in rats by Crawford (4). In man, the experiments of Epstein, Kleeman, Pursel and Hendrikx (5) showed that urine concentrating ability is decreased on a low-protein diet.

All these studies, as well as those presented in this paper, lead to the conclusion that urea has a unique role in the concentrating mechanism. In the preceding paper (6) and elsewhere (7), an hypothesis has been presented which accounts for this role of urea. It is postulated that the passive accumulation of urea in the medullary interstitial fluid from the collecting ducts permits urea to add to the osmotic pressure of the urine without being balanced by an equivalent amount of sodium chloride actively transported into the medullary interstitium. The data in this paper confirm several predictions derived from this hypothesis.

In an animal on a low protein diet, the filtered load of urea is decreased. The amount of urea reaching the collecting ducts is presumably also greatly diminished. If accumulation of urea from the ducts in the medullary interstitium enhances urine concentration, maximum urine osmolality would be expected to fall when a low protein diet is given. The magnitude of the fall in urine osmolality due to changes in medullary urea should be approximately equal to the change in urinary urea concentrations. In these studies on the dog, the decrease in urine urea concentration accounts for some 80 per cent of the decrease in urine osmolality. Because of the change in osmolality and urea concentration as urine passes through the ureters and bladder at low flows (10) [also see footnote to Results in preceding paper (6)], it is difficult to be certain whether the change in urine urea concentrations in the kidney actually accounts for the entire change in urine osmolality on the low protein diet in the dog, or whether other factors must be considered. In man (5), the urine flows remained much higher and the decrease in urine urea concentration accounted for a large fraction of the decrease in osmolality in all but two subjects, in whom only small decreases in urinary urea concentration occurred.

If the decrease in urine osmolality on a low protein diet is in large part due to the fall in urinary urea, one would predict that maximum urine concentration would increase abruptly when urea is given to an animal on a low protein diet. The data in Tables III and IV show that this is true in both the dog and in man. The increase in urinary urea concentration accounts for the greatest part of the increased urinary osmolality. In the human studies, agreement between changes in urea concentration and osmolality was so close as to require no other factors to be postulated to account for the change. In the dog, the change in urea apparently accounts for about 80 per cent of the increase in osmolality. Because of the changes in urine composition in the lower urinary tract mentioned above, it is difficult to be sure how close

the agreement actually is between the changes in urea and the osmolality of urine as it left the kidney. In the studies of Epstein and co-workers (5), urea was found to have a chronic but not an acute effect on urine concentration in humans on low protein diets. In the experiment illustrated, urine osmolality remained constant in the face of a twofold increase in solute excretion, which with other solutes would be expected to result in a fall in urine osmolality. The acute dose of urea was about three times that given in the present experiments. Probably the effects of a somewhat greater solute diuresis in the studies of Epstein and associates (5) masked the rise in urine concentration which otherwise would have occurred.

The maximum negative free-water clearance falls in dogs and man (5) on low protein diets. However, it is difficult to interpret and to quantify the changes in dogs reported in the present studies. In these experiments, and in a number of other unpublished studies done in dogs on regular diets for various purposes, $T^c_{H_2O}$ did not regularly approach a maximum as solute excretion was increased by mannitol infusions. Instead, $T^c_{H_2O}$ usually first increased, then decreased as solute excretion was raised.[1] It was therefore not possible to determine whether urea has an acute effect on $T^c_{H_2O}$. The present hypothesis would predict that the $T^c_{H_2O}$ would be greater during mannitol diuresis on a regular diet, to the extent that urine urea concentrations are greater. The data in Table II show that at comparable levels of solute excretion urea concentrations are higher on a regular diet than on a low protein diet. A small increase in urine urea concentration (balanced by urea in the interstitial fluid of the medulla) would substantially increase $T^c_{H_2O}$ at high flows. For example, if 50 μM per ml. of urea were added when urine flow is 8 ml. per minute and $T^c_{H_2O}$ is 2 ml. per minute $T^c_{H_2O}$ would increase to about 3.3 ml. per minute. In two dogs it was found that during infusion of urea the negative free-water clearance is higher at all levels of solute excretion than that found during mannitol infusion. This was true both on high and on low protein diets. (However, the increase in $T^c_{H_2O}$ was in proportion to the increase in GFR which regularly occurs when urea is given as compared to mannitol.) In man (5), no acute effect of urea on $T^c_{H_2O}$ was found.

If urea accumulates in the medulla by passive movement from the collecting ducts, the concentration of urea in medullary tissue water cannot be greater than that in the urine. In the dogs on low protein diets, the urea concentration in the tissue water for the papilla was equal to or somewhat greater than that of the urine. However, as was discussed above and in the preceding paper (6), urine urea concentrations decrease up to 40 per cent when urine is perfused through the ureter and bladder at flows comparable to those seen in dehydrated dogs on low protein diets. This factor probably accounts for the greater tissue than urine concentrations in these dogs. Schmidt-Nielsen (12) has postulated that urea is actively transported in a counter-current multiplier mechanism in the medulla. In our opinion all the data in the dog can be explained without invoking active movement of urea. In addition, the explanation of the low medullary urea concentrations during water diuresis [(13) and unpublished observations] in the presence of an active transport mechanism requires either that the activity of the mechanism be graded according to urine flow, or that the flow take place through wholly cortical nephrons and capillaries in water diuresis (11). A much simpler explanation, requiring no *ad hoc* hypotheses, is that tissue urea concentration is low because the urine urea concentration is low. According to the hypothesis of passive accumu-

[1] Positive free-water clearances (urine hypotonic to plasma) were common, even in dehydrated dogs receiving large doses of vasopressin, when the excreted solute was 10 to 15 per cent or more of the filtered solute. At low solute excretions, the tubular fluid becomes isosmotic with plasma in the distal convoluted tubule, when antidiuretic hormone is present (11). The finding that urine is dilute to plasma at moderately high solute loads presumably indicates incomplete osmotic equilibration of tubular fluid in the distal convoluted tubule. The observed $T^c_{H_2O}$ at moderate and high solute loads in these dogs thus probably represents the difference between the "positive free-water" present at the end of the distal convoluted tubule and the net reabsorption of free-water ("negative free-water") at the concentrating site in the collecting ducts. Only if the degree of residual disequilibrium at the end of the convoluted tubule is the same in the dog on high and low protein diets is the appearance of net "positive free-water" at lower solute excretions in the dog on a low protein diet an indication of the decrease in net water reabsorption at the concentrating site.

lation of urea, the urea concentration in the medulla cannot exceed that in the urine.

In the preceding paper, it was shown that acute changes in glomerular filtration rate under certain circumstances are associated with a fall in urine concentration. Most of this decrease could be explained as due to a decrease in delivery of urea to the medulla. This part of the fall in osmolality would be analogous to the falls reported in this paper on low protein diets, though the ultimate cause of decreased delivery of urea to the medulla is, of course, quite different. In addition, a decrease in sodium concentration in the medulla was sometimes found during acute reduction of the GFR at low solute excretions. A chronic reduction of GFR occurred in most of the dogs maintained on a low protein diet. However, the sodium concentrations in the medulla (Table V) are within the range usually seen in dehydrated dogs on regular diets in our studies. At least by this rather gross measure, active sodium transport into the medullary interstitial fluid does not seem to be decreased on a low protein diet.

The data in this paper are consistent with the hypothesis that the "economy of water in renal function referable to urea" (1) is the result of the passive accumulation of urea in the medullary interstitial fluid. Such an economy is a particularly favorable adaptation, since in mammals urea is the principal waste product requiring water for excretion in the urine.

SUMMARY

1. The maximum urine osmolality and the maximum negative free-water clearance are reduced in dogs on low protein diets. Eighty per cent or more of the decrease in osmolality can be accounted for by the decrease in the urea concentration of the urine.

2. In both the dog and man, the administration of urea to individuals on a low protein diet results in an abrupt increase in maximum urine osmolality. Eighty per cent or more of this increase in the dog, and essentially all of the increase in man, can be accounted for by the increased urea concentration in the urine.

3. Renal medullary tissue urea concentration in the dog on a low protein diet is markedly reduced, but tissue sodium concentration is unchanged.

4. These observations support the hypothesis that the passive accumulation of urea in the medullary interstitial fluid increases the maximum urine concentrations which the kidney can attain, and that urea therefore has a unique role in the urine concentrating mechanism.

REFERENCES

1. Gamble, J. L., McKhann, C. F., Butler, A. M., and Tuthill, E. An economy of water in renal function referable to urea. Amer. J. Physiol. 1934, **109**, 139.
2. Kellogg, R. H., and Koike, T. I. Difference between mannitol and urea diuresis in the rat (abstract). Amer. J. Physiol. 1955, **183**, 633.
3. Radford, E. P., Jr. Factors modifying renal concentrating mechanism in rats. Fed. Proc. 1958, **17**, 127.
4. Crawford, J. D., Doyle, A. P., and Probst, J. H. Service of urea in renal water conservation. Amer. J. Physiol. In press.
5. Epstein, F. H., Kleeman, C. R., Pursel, S., and Hendrikx, A. The effect of feeding protein and urea on the renal concentrating process. J. clin. Invest. 1957, **36**, 635.
6. Levinsky, N. G., Davidson, D. G., and Berliner, R. W. Effects of reduced glomerular filtration on urine concentration in the presence of antidiuretic hormone. J. clin. Invest. 1959, **38**, 730.
7. Berliner, R. W., Levinsky, N. G., Davidson, D. G., and Eden, M. Dilution and concentration of the urine and the action of antidiuretic hormone. Amer. J. Med. 1958, **24**, 730.
8. Berliner, R. W., and Davidson, D. G. Production of hypertonic urine in the absence of pituitary antidiuretic hormone. J. clin. Invest. 1957, **36**, 1416.
9. Smith, H. W. Principles of Renal Physiology. New York, Oxford University Press, 1956, p. 121.
10. Levinsky, N. G., and Berliner, R. W. Amer. J. Physiol. In press.
11. Wirz, H. The location of antidiuretic action in the mammalian kidney *in* The Neurohypophysis, H. Heller, Ed. New York, Academic Press Inc., 1957, p. 157.
12. Schmidt-Nielsen, B. Urea excretion in mammals. Physiol. Rev. 1958, **38**, 139.
13. Ullrich, K. J., and Jarausch, K. H. Untersuchungen zum Problem der Harnkonzentrierung und Harnverdünnung. Pflüg. Arch. ges. Physiol. 1956, **262**, 537.

TOXICITY OF UREA AND ITS ROLE IN THE PATHOGENESIS OF UREMIA *

By EVELYN FRANCES GROLLMAN and ARTHUR GROLLMAN

(From the Department of Experimental Medicine, University of Texas Southwestern Medical School, Dallas, Texas)

(Submitted for publication October 17, 1958; accepted January 5, 1959)

It is generally assumed that urea is nontoxic and that even the high concentrations present in uremia are innocuous and do not contribute to the symptoms encountered in this condition. In support of this assumption is the fact that the blood in the elasmobranch fishes contains 330 to 440 mM (about 2 per cent) of urea per L. The original purpose of the present study was to utilize this assumed inertness of urea in order to determine the effect of the presence of one molecular species in high concentration on the electrolyte and water composition of the body. However, it soon became apparent that urea in high concentrations, contrary to the generally accepted notion, was lethal and that many of the abnormalities noted in uremia could be elicited by this substance.

METHODS

General procedure. Intermittent peritoneal lavage, as described elsewhere (1), was utilized to maintain the desired level of urea in the extracellular fluid of the nephrectomized dog. The kidneys were removed under ether anesthesia through the dorsolumbar approach with an interval of 7 to 14 days between the two operations. Sterile solutions available commercially (Peridial®)[1] or prepared as described previously (1) were utilized for the peritoneal exchanges. One L. of the solution, the electrolyte and water composition of which approximated normal extracellular fluid, was injected intraperitoneally and exchanged, twice daily (2). Varying amounts of urea (5 to 30 Gm. per L.) were added to these solutions in order to maintain the concentration at the desired level. The animals were killed by exsanguination after 4 to 10 days, at a time when they manifested severe symptoms of uremia.

Analytical procedures. The tissues were placed in dry, glass-stoppered weighing bottles and dried *in vacuo* at 40° C. to constant weight. They were then defatted with petroleum ether, dried again, reweighed and extracted with a known volume of 0.75 N HNO_3. Urea was determined, after neutralization of the tissue extract, by the urease procedure of Van Slyke and Cullen (3); chloride, as described by Laramore and Grollman (4); and sodium and potassium by a Beckman flame photometer.

The urea used in the present study was a chemically pure preparation (Merck Reagent Crystals). The injection intraperitoneally of a liter of a 5 per cent solution of this preparation induced no pyrogenic or other obvious toxic reaction such as has been reported to follow the injection intravenously of commercially available preparations of urea in the human. Intact dogs treated with large amounts of urea for as long as a month revealed no symptoms of toxicity, but the rapid excretion of the urea precluded their use for the purpose of the present study.

RESULTS

General symptoms

The period of survival of the six dogs used in the present study and other pertinent data are summarized in Table I. As noted in Column 2, the animals were maintained for periods of four to nine days. One animal died after four days; another after seven days; the others were killed five to nine days after initiating the peritoneal dialysis, at which time they were drowsy or comatose and the urea content of their blood serum had attained the values shown in Column 4 of the table.

Symptoms of intoxication were apparent clinically two to four days after beginning the dialysis, at which time the blood urea content was 370 to 480 mg. per cent. The first symptoms noted were weakness and anorexia, the animals refusing their food, manifesting no interest in their surroundings and lying inert, in contrast to the normal behavior of animals maintained with only moderately elevated blood urea levels (2). Vomiting and retching soon followed and persisted, with diarrhea terminating in hemorrhage from the bowel. The body temperature remained normal for three or four days but then gradually declined to 90 to

* This work was supported in part by the Dixie and Grady Vaughn Fund.

[1] Kindly supplied through the courtesy of Dr. E. B. McLean, Research Director, The Cutter Laboratories, Berkeley, Cal.

TABLE I

Effects of an elevated urea content of the blood in the nephrectomized dog

Dog	Duration of experiment	Time of onset of symptoms	Urea content of blood at onset of symptoms	Urea content of blood at termination of experiment	Condition of dog at termination of experiment
no.	*days*	*days*	*mg. %*	*Gm./L.*	
1	8	4	430	16.9	Comatose
2	4	2	480	14.4	Dead
3	6	3	370	15.9	Drowsy
4	9	2½	380	9.0	Drowsy
5	7	2¼	410	8.6	Dead
6	5	3	450	5.4	Drowsy

TABLE II

The effect of a high urea level of the blood on the water and electrolyte content of the tissues of Dog No. 1 (cf. Table I)

Tissue	Urea	Water	Sodium	Potassium	Chloride
	Gm./Kg. H_2O	*Gm./Kg.*	*mEq./Kg.*	*mEq./Kg.*	*mEq./Kg.*
Blood serum	16.9	912	136	5.2	94
Heart	16.7	757	48	99	35
Liver	17.7	727	37	102	25
Skeletal muscle	17.0	742	64	83	23
Spleen	18.0	748	65	94	35
Stomach	17.0	755	56	76	49

95° F. (rectally) when the experiment was terminated. Symptoms gradually increased in intensity and culminated finally with the animals in a state of deep torpor or coma.

Biochemical changes

The urea, water and electrolyte content of the tissues at the termination of the experiments are shown in Tables II through VII. All values are

TABLE III

The effect of a high urea level of the blood on the water and electrolyte content of the tissues of Dog No. 2 (cf. Table I)

Tissue	Urea	Water	Sodium	Potassium	Chloride
	Gm./Kg. H_2O	*Gm./Kg.*	*mEq./Kg.*	*mEq./Kg.*	*mEq./Kg.*
Blood serum	14.4	915	140	4.6	101
Heart	14.3	766	49	80	28
Liver	14.4	736	41	90	30
Skeletal muscle	14.5	748	67	92	26
Spleen	14.9	750	68	96	40

TABLE IV

The effect of a high urea level of the blood on the water and electrolyte content of the tissues of Dog No. 3 (cf. Table I)

Tissue	Urea	Water	Sodium	Potassium	Chloride
	Gm./Kg. H_2O	*Gm./Kg.*	*mEq./Kg.*	*mEq./Kg.*	*mEq./Kg.*
Blood serum	15.9	910	140	5.1	101
Heart	15.9	760	50	98	36
Liver	16.8	730	41	94	28
Skeletal muscle	16.4	750	69	86	26
Spleen	16.6	754	71	90	32
Stomach	16.0	760	58	70	51

TABLE V

The effect of a high urea level of the blood on the water and electrolyte content of the tissues of Dog No. 4 (cf. Table I)

Tissue	Urea	Water	Sodium	Potassium	Chloride
	Gm./Kg. H_2O	*Gm./Kg.*	*mEq./Kg.*	*mEq./Kg.*	*mEq./Kg.*
Blood serum	9.0	905	130	5.0	93
Heart	9.0	780	38	81	27
Liver	9.0	786	36	98	31
Skeletal muscle	9.6	764	23	96	24
Spleen	9.9	753	44	90	33
Stomach	9.2	758	37	74	44

expressed in terms of fat-free wet tissue except for urea and water which are expressed in terms of the water content and fresh wet-weight of the tissues, respectively. As noted in the second column of the tables, the urea, as anticipated, diffuses throughout the body water and is present in the tissue water in a concentration equal to, and in some instances slightly greater than, that present in the extracellular water. The differences noted, however, with the exception of the spleen, are slight.

That the effects reported in Tables II through VII are not secondary to electrolyte or acid-base disturbances induced by intermittent peritoneal lavage is shown in Table VIII in which the urea, sodium, potassium, chloride and bicarbonate contents of the blood are given after equilibration with urea had occurred. As demonstrated previously (1, 2) the exchange of solutes across the peritoneal membrane conforms to the physicochemical laws of distribution across a semipermeable membrane. The composition of the lavage fluid is such as to maintain a relatively normal electrolyte and acid-base balance in the extracellular fluid in the absence of the kidneys.

In Table IX are reproduced data on tissue analyses on dogs maintained by intermittent peritoneal lavage following bilateral nephrectomy as compared to normal control animals. The observed deviations, which are minor as compared to

TABLE VI

The effect of a high urea level of the blood on the water and electrolyte content of the tissues of Dog No. 5 (cf. Table I)

Tissue	Urea	Water	Sodium	Potassium	Chloride
	Gm./Kg. H_2O	*Gm./Kg.*	*mEq./Kg.*	*mEq./Kg.*	*mEq./Kg.*
Blood serum	8.6	910	138	5.1	101
Heart	8.6	790	46	83	25
Liver	8.8	795	39	95	33
Skeletal muscle	9.0	770	24	99	26
Spleen	9.3	761	36	92	35
Stomach	8.8	768	35	70	40

TABLE VII

The effect of a high urea level of the blood on the water and electrolyte content of the tissues of Dog No. 6 (cf. Table I)

Tissue	Urea	Water	Sodium	Potassium	Chloride
	Gm./Kg. H_2O	*Gm./Kg.*	*mEq./Kg.*	*mEq./Kg.*	*mEq./Kg.*
Blood serum	5.4	908	140	4.9	99
Heart	5.4	775	42	86	24
Liver	5.5	790	41	88	34
Skeletal muscle	5.6	760	30	101	29
Spleen	5.7	770	39	98	30
Stomach	5.5	762	34	81	48

TABLE VIII

The electrolyte content of the blood plasma of bilaterally nephrectomized dogs subjected to intermittent peritoneal lavage with solutions containing various urea concentrations

Dog	Days after nephrectomy	Urea	Sodium	Potassium	Chloride	Bicarbonate
no.		*Gm./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*
1	5	14.9	142	4.6	100	20.0
2	4	14.4	140	4.6	101	21.2
3	5	15.6	145	4.3	98	22.0
4	7	8.6	143	4.4	103	20.1
5	6	8.0	141	4.2	105	18.9
6	5	5.4	140	4.9	99	23.4

those obtained in the present study, may be attributed to the increased extracellular fluid volume and the hypertension induced by nephrectomy.

Urea is present in blood and other aqueous solutions as a dimer (5) but is apparently not bound by the blood proteins to any appreciable extent. When added in a concentration of 3 per cent to blood serum it was found to be completely filtrable through a cellophane membrane. The unequal distribution of urea between the plasma and red blood cell as found by Ralls and Crohn (6) and between the intracellular and extracellular fluid in the present studies may be attributed either to binding on some cellular component or to partial dissociation of the urea dimer within the cell (5).

There was, in general, a reduction of the water content of the tissues which was reflected in dehydration of the tissue as noted at autopsy. Although this may be secondary to the increased total osmotic pressure of the tissue fluids due to their high urea content, it more probably reflects the anorexia, vomiting and reduced water intake of the animals.

As noted in the last three columns of Tables V through VII, the electrolyte pattern of the blood and tissues is not materially altered by the presence of 150 mM of urea per L. in the body fluids. However, when the urea content is increased to 280 mM, as in Tables II through IV, there is an increase in the sodium and chloride contents with a corresponding decrease in the potassium contents of certain tissues, particularly the skeletal muscle and spleen. These changes reflect the toxic effects of urea superimposed on the effects of nephrectomy which induces similar effects of a lesser degree in animals maintained by intermittent peritoneal lavage as in the present experiments (7).

TABLE IX

Water and electrolyte contents of the tissues of nephrectomized dogs maintained by intermittent peritoneal lavage (experimental) as compared to normal (control) animals *

Tissue	Water	Sodium	Potassium
	Gm./Kg.	*mEq./Kg.*	*mEq./Kg.*
Brain			
Control	775 ± 2.8	56.9 ± 1.75	98.4 ± 0.90
Experimental	778 ± 16.6	59.3 ± 1.85	97.5 ± 6.54
Heart			
Control	772 ± 22.0	40.1 ± 2.14	84.8 ± 2.38
Experimental	779 ± 26.0	42.5 ± 4.25	84.1 ± 8.52
Liver			
Control	707 ± 18.2	36.4 ± 1.9	89.9 ± 1.17
Experimental	710 ± 16.2	39.5 ± 5.2	90.1 ± 2.15
Skeletal muscle			
Control	762 ± 2.3	26.7 ± 1.4	112.2 ± 1.23
Experimental	768 ± 3.1	29.2 ± 2.1	110.4 ± 10.05
Spleen			
Control	777 ± 2.5	31.6 ± 0.2	115.5 ± 1.10
Experimental	769 ± 14.8	33.5 ± 1.4	112.2 ± 2.40
Gut			
Control	772 ± 22.0	43.5 ± 1.00	103.1 ± 1.11
Experimental	780 ± 35.4	41.0 ± 5.65	89.1 ± 8.46

* The results represent the average values obtained on a series of six animals.

DISCUSSION

The procedure used in the present study permits one to dissociate the effect of a high urea level from that induced by the other abnormalities which accompany renal insufficiency with nitrogen retention and give rise to the clinical pattern designated as "uremia." Dogs maintained on intermittent peritoneal lavage, as used in the present study, but without the addition of urea to the dialysis fluid, may be maintained in apparently normal condition for several months except for the

development of hypertension (2). Since the only variable introduced in the present experiments was a high urea content of the extracellular fluid, the observed abnormalities in the clinical state of the animals must be attributed to the elevated urea.

Although many of the symptoms of "uremia" are undoubtedly attributable to acidosis, electrolyte-water imbalance and other accompaniments of renal insufficiency, some of the toxic manifestations are apparently due to the accumulation of urea, rather than to the creatinine, uric acid or other unknown products of catabolism, as assumed hitherto.

Although the concentrations of urea used in some of the experiments were purposely elevated beyond that normally observed, toxic effects were noted at concentrations encountered clinically and it may be inferred that even lower concentrations might, if maintained for prolonged periods, induce equally noxious effects.

The earliest and most characteristic symptoms of "uremia" are weakness and anorexia. This was also the first symptom observed in the present study and was elicited at blood levels of urea comparable to those seen in severe renal insufficiency in the human. Nausea and vomiting was a constant feature in our dogs as in the human subject.

Although such neurological disturbances as headache or abnormal sensations cannot be detected in the experimental animal, disturbances in the mental state with coma terminally were apparent. Diarrhea was an early symptom, culminating in ulcerative colitis and hemorrhage from the bowel. On the other hand, the convulsions and twitching common in the uremic patient were not observed in the experimental animal and are probably cerebral in origin and secondary to alterations in salt and water metabolism. Itching was likewise not observed in the present experiments and may be attributed to the fact that, unlike the human, sweating does not occur in the dog and hence there is no accumulation of urea on the skin.

Since acidosis, water-electrolyte balance and the accumulation of other catabolites was avoided by the procedure used in the present experiments, one must attribute the observed symptoms to the presence of urea in high concentrations and conclude that, contrary to generally accepted notions, this substance is noxious when presented chronically to the tissues in high concentration as in terminal renal insufficiency.

The observed toxicity of urea is not surprising if one considers the capacity of this agent to react with the vital components of the cell. Its noxious effects may be attributed either to its specific action on the cell or to the hypertonicity induced by its presence in high concentration throughout the body fluids. A direct action of urea on certain components of the cell may result from its demonstrated affinity for proteins with disruption of vital enzyme systems. Thus, urea has been shown to combine with heme (8), plasma albumin (9) and sodium deoxyribonucleate (10) and to induce denaturation (11) or disruption of the molecule (10). Although the concentrations of urea required to demonstrate these effects *in vitro* are of a much greater magnitude than those used in the present experiments, some reactions would be anticipated to occur at lower concentrations which would account for the present observations.

The failure of previous attempts to demonstrate the toxicity of urea is explained by the fact that the methods used were inadequate for maintaining a high concentration of urea in the tissues for prolonged periods. Because of its ready diffusibility and rapid elimination by the kidney, the injection of even large doses results only in a brief concentration of urea in the tissues. However, in rabbits with ligated ureters the injection of 2 Gm. of urea per Kg. of body weight has been reported to induce death within one hour with an accumulation of urea in variable amounts in certain tissues (12). The disruptive effects of urea on the electrolyte content of the tissues demonstrated in the present study may in part account for the similar dislocations observed in acute uremia (13).

SUMMARY

High concentrations of urea (540 to 1,690 mg. per cent) were maintained in the extracellular fluid of nephrectomized dogs by means of intermittent peritoneal lavage. This technique permits the maintenance of the other components of the extracellular fluid constant and thus allows one to study the specific effects of urea. High concentrations of urea induce many of the symptoms encountered in uremia which have been attributed hitherto to changes other than the accumulation of urea.

Of the various symptoms observed in the late stages of chronic renal failure, it would appear from the present observations that many of these previously attributed to some unknown catabolite accumulating in the uremic patient may be due to the effect of the accumulated urea previously looked upon as an innocuous agent. The presence of high urea levels also induces certain dislocations of the electrolyte composition of the tissues which are responsible for, or at least are accompaniments of, the disruptive effects of urea on the components of the tissue cells to which presumably it owes its toxicity.

REFERENCES

1. Grollman, A. Acute Renal Failure. Springfield, Ill., Chas. C Thomas, 1954, p. 63.
2. Grollman, A., Turner, L. B., and McLean, J. A. Intermittent peritoneal lavage in nephrectomized dogs and its application to the human being. A.M.A. Arch. intern. Med. 1951, **87**, 379.
3. Van Slyke, D. D., and Cullen, G. E. A permanent preparation of urease, and its use in the determination of urea. J. biol. Chem. 1914, **19**, 211.
4. Laramore, D. C., and Grollman, A. Water and electrolyte content of tissues in normal and hypertensive rats. Amer. J. Physiol. 1950, **161**, 278.
5. Grollman, A. The vapour pressures of aqueous solutions with special reference to the problem of the state of water in biological fluids. J. gen. Physiol. 1931, **14**, 661.
6. Ralls, J. O., and Crohn, E. B. Urea is not equally distributed between the water of the blood cells and that of the plasma. J. biol. Chem. 1943, **151**, 529.
7. Grollman, A. Water and electrolyte content of the tissues of the adrenalectomized and adrenalectomized-nephrectomized dog. Amer. J. Physiol. 1954, **179**, 36.
8. Burk, N. F., and Greenberg, D. M. The physical chemistry of the proteins in non-aqueous and mixed solvents. I. The state of aggregation of certain proteins in urea-water solutions. J. biol. Chem. 1930, **87**, 197.
9. Huggins, C., Tapley, D. F., and Jensen, E. V. Sulphydryl-disulphide relationships in the induction of gels in proteins by urea. Nature (Lond.) 1951, **167**, 592.
10. Mathieson, A. R., and Porter, M. R. The effect of urea on the streaming birefringence and viscosity of sodium deoxyribonucleate. J. chem. Soc. 1958, 1298.
11. Ruffo, A., Santamaria, R., and Mattace-Raso, F. The denaturation of serum proteins by urea. Boll. Soc. ital. Biol. sper. 1955, **31**, 1381.
12. Schiano, S., and D'Onofrio, F. Experimental uremia. Residual and urea nitrogen. Sperimentale 1957, **107**, 435.
13. Hamburger, J. Electrolyte disturbances in acute uremia. Clin. Chem. 1957, **3**, 332.

SIALIC ACID IN HUMAN SEMEN AND IN THE MALE GENITAL TRACT

By LEONARD WARREN

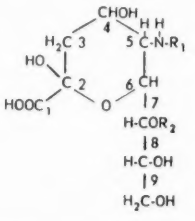
(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda Md.)

(Submitted for publication October 28, 1958; accepted January 9, 1959)

There has been in recent years a growing interest in a family of compounds which has the group name sialic acid (1). These aminosaccharides have in common a chain of nine carbon atoms called neuraminic acid (Figure 1). Neuraminic acid itself has not been found naturally, but certain acetyl and glycolyl derivatives have been detected in many of the tissues and fluids of mammals (2, 3) and in bacteria (4). Neuraminic acid may bear one acetyl (N-acetylneuraminic acid) or one glycolyl group (N-glycolylneuraminic acid) on the amino-nitrogen atom, or it may bear two acetyl groups (N,O-diacetylneuraminic acid). In the latter compound the second acetyl moiety is believed to be in ester linkage with the hydroxyl of carbon number seven of neuraminic acid. The structure of these compounds has been established by various workers and N-acetylneuraminic acid has been synthesized by chemical (5) and enzymatic (6) means. Very little sialic acid is found free in tissues and secretions. Most is bound to the carbohydrate portion of mucoproteins and can be released by relatively mild acid hydrolysis (2), or by enzmatic action (7, 8).

The only data in the literature on seminal sialic acid is a note by Odin (9) on the presence of sialic acid in the gelatinous fragments of hog semen. While our study was in progress, Svennerholm (2) published a new method of analysis for sialic acid in biological materials. The sialic acid content of a number of tissues and secretions was given, among which was an average sialic acid concentration of 105.5 mg. per 100 Gm. of human semen (four specimens).

As part of a study on the role of sialic acid in fertilization, we have assayed a series of normal and subnormal human semen specimens as well as human prostatic and seminal vesicle secretions and spermatozoa for their content of sialic acid. A study has been made of the sialic acid concentration in semen determined by colorimetric methods directly on semen, and by Svennerholm's method which entails partial purification of sialic acid by an anion exchange column.



		R_1	R_2
Neuraminic acid		H	H
Sialic acid	N-acetylneuraminic acid	$-\overset{O}{\overset{\|}{C}}-CH_3$	H
	N-glycolylneuraminic acid	$-\overset{O}{\overset{\|}{C}}-CH_2OH$	H
	N,O-diacetylneuraminic acid	$-\overset{O}{\overset{\|}{C}}-CH_3$	$-\overset{O}{\overset{\|}{C}}-CH_3$

FIG. 1. THE STRUCTURE OF NEURAMINIC ACID AND ITS NATURALLY OCCURRING DERIVATIVES

MATERIALS

Semen samples were obtained from individuals who were being investigated for possible infertility.[1] Semen analysis was part of a general examination to investigate the cause of a barren marriage. In most cases colorimetric analysis for sialic acid was carried out within eight hours of ejaculation. There was no decrease in the sialic acid content of semen when it was kept at 2° C. for two weeks. Semen for other studies was stored at −15° C.

Human seminal vesicle and its secretion, prostate and testes were obtained from autopsies carried out in the Pathologic Anatomy Branch of the National Cancer Institute. Normal prostatic secretions were obtained from out-patients at the Urology Clinic of the United States

[1] We wish to thank Dr. M. E. Voss of the Oscar B. Hunter Laboratory and Drs. E. E. Ferguson and F. T. Reuter of Washington, D. C. for obtaining this material for us and for carrying out the sperm count and microscopic examination.

Naval Hospital, Bethesda, Maryland.[2] Samples were cooled to 0° C. immediately and analyzed within 1.5 hours.

N-Acetylneuraminic acid was chemically synthesized by the method of Cornforth, Firth and Gottschalk (5). Sialic acid, presumably N,O-diacetylneuraminic acid, was isolated from bovine submaxillary gland mucin, by the method of Blix, Lindberg, Odin and Werner (10).

METHODS

Ehrlich reaction. All samples of semen were routinely assayed for sialic acid by the direct Ehrlich method of Werner and Odin (11). The assay was modified by reducing the sample size and reagent volume to one-tenth that used by Werner and Odin. Semen was vigorously shaken to attain homogeneity and 0.1 ml. diluted to 1 ml. with distilled water. Aliquots (0.1 ml. and 0.2 ml.) of diluted semen were transferred to test tubes (10×125 mm.) and the volume was adjusted to 1 ml. with distilled water. A blank consisted of 1 ml. distilled water. Ehrlich's reagent (0.2 ml.) was then added (11), the tubes were shaken, capped with glass marbles and placed in a vigorously boiling water bath for exactly 30 minutes. The tubes were cooled in water at room temperature for seven minutes and then centrifuged for four minutes in a clinical centrifuge. The clear solutions were pipetted into micro-cuvettes 3 mm. wide, with a light path of 1 cm., and optical densities were determined at 565 mμ, in a Beckman DU spectrophotometer, equipped with a micro-attachment. Optical density readings were made against the blank.

Two concentrations of semen were used in each assay to ensure that there was no deviation from linearity at the sialic acid levels used. The optical density value produced by 0.2 ml. of the diluted semen (0.02 ml. of the original semen sample) was used to calculate the sialic acid concentration. In order to obtain sialic acid concentrations in mg. per 100 ml. semen, the observed optical density at 565 mμ was multiplied by a factor of 1,130. A molar extinction coefficient of 1.640×10^2 was used. The sialic acid present is most probably N-acetylneuraminic acid with a molecular weight of 309. Variation of ± 3 per cent from the average was observed in six analyses of one semen specimen over the course of a month.

Sialic acid was also assayed by the orcinol method of Werner and Odin (11), by the resorcinol method of Svennerholm (12), and by the diphenylamine procedure of Pigman, Hawkins, Blair and Holley (13).

Anion exchange resin method. A few small modifications of Svennerholm's method were made (2). One-tenth of a ml. of semen was hydrolyzed at 80° C. in 0.1 N sulfuric acid for one hour. The samples were cooled and placed on columns of Dowex-1-acetate (5×0.8 cm.) (X-8, 100-200 mesh). The hydrolysis tubes and columns were washed twice with 5 ml. of water and the sialic acid was then eluted into a 10 ml. graduated cylinder with 9 ml. of 1 M sodium acetate buffer, pH 4.6. Water was added to the graduated cylinders to the 10 ml. mark. To 0.2 and 1.0 ml. of eluate in 10×125 mm. test tubes, water was added to a volume of 1 ml. The blank consisted of 1 ml. 1 M acetate buffer, pH 4.6. One ml. of resorcinol reagent was added, a bead was placed on top of the tube and the tubes were shaken and then heated in a vigorously boiling water bath for exactly 15 minutes. The tubes were cooled in running water for 10 minutes and 1.0 ml. of isoamyl alcohol was added to each tube. The tubes were shaken vigorously for 20 seconds and centrifuged for 5 minutes in a clinical centrifuge. The clear top phase of isoamyl alcohol was pipetted into cuvettes, 3 mm. wide with a 1 cm. light bath. The optical density at 580 mμ was observed in the Beckman DU spectrophotometer equipped with a micro-attachment. In this assay 1 μM of N-acetylneuraminic acid in a volume of 1 ml. gave an optical density reading at 580 mμ of 4.650. Repeated analyses of semen samples agreed within 2 per cent.

Control assays were run with 0.2 and 0.3 μM of N-acetylneuraminic acid. This is approximately the amount in the semen specimens assayed. A recovery of 91.5 to 94 per cent was observed and the average loss of N-acetylneuraminic acid in seven assays was 7.3 per cent, which is comparable to the figures of Svennerholm (2). We have, therefore, increased our observed sialic acid values by 7.3 per cent to compensate for sialic acid which is either destroyed by acid hydrolysis or is lost on the column.

Experiments have shown that after acid hydrolysis of semen the precipitate formed contains no sialic acid. All of the sialic acid is rendered free and dialyzable. After elution of sialic acid from the column with 9 ml. of 1 M acetate buffer no further sialic acid could be eluted, even with 3 N HCl.

Isolation of sialic acid. Sialic acid was isolated from 3 Gm. each of seminal vesicle, prostate, and testis by heating homogenates of these tissues in 15 ml. 0.05 N sulfuric acid for two hours at 80° C. Sialic acid was also isolated from 10 ml. semen by adjusting the pH to 1.0 with sulfuric acid, and then heating in the above manner. The suspensions were centrifuged at $12{,}000 \times G$ for 20 minutes and the supernatant solutions adjusted to pH 8.0 with a 1 molar solution of NaOH. The solutions were placed on a Dowex-1-formate column (X-8, 100-200 mesh) (10×0.8 cm.) and washed with 50 ml. distilled water, 30 ml. 0.05 M formic acid, and then eluted with 15 ml. 0.3 M formic acid.

The eluates containing sialic acid were then placed on columns of Norit A-Hyflo Super-cel (Johns Manville), 1:4 (6×0.8 cm.). These were washed with 30 ml. of water and the sialic acid was eluted with 20 ml. of 40 per cent aqueous methanol. The methanol eluates were evaporated to dryness *in vacuo*. The material obtained was used for paper chromatography experiments and chemical tests.

Paper chromatography. The descending method was used with Whatman No. 1 paper at room temperature

[2] We wish to thank Capt. J. S. Hanten, M. C., U.S.N., Department of Urology, for obtaining this material.

for 18 hours. The solvent systems were: *1*) *n*-butanol: acetic acid: water (4:1:5); *2*) *sec*-butanol: acetic acid: water (4:1:5); *3*) ethyl acetate: pyridine: water (1:1:2); *4*) n-butanol: *n*-propanol: 0.1 N HCl, (1:2:1) (10, 14). To locate the sialic acid, the entire paper strip was cut into sections 1 cm. × 2 cm. wide and placed in 15 × 125 mm. test tubes which contained 1 ml. distilled water and 0.2 ml. Ehrlich's reagent. The usual assay for sialic acid was carried out.

Neuraminidase and semen. The following experiment was performed to see whether the enzyme neuraminidase could cleave sialic acid from seminal mucoprotein, as it does from certain human plasma proteins (8).

One-tenth of a ml. of semen was incubated with 10 μg. of neuraminidase and 50 μM of sodium acetate buffer, pH 5.25, in a final volume of 0.75 ml. After 24 hours, 0.75 ml. of 5 per cent phosphotungstic acid in 2 N HCl was added to the vessels to precipitate the mucoprotein and the vessels were centrifuged. In this experiment, free, nonprecipitable sialic acid, released by the enzyme, was assayed by the orcinol reaction. This assay was carried out on 1 ml. of clear supernatant solution. In the control vessel, enzyme was omitted and no orcinol reacting material was released.

The neuraminidase used was derived from the culture filtrate of *Clostridium perfringens* (8) and purified 12-fold by adsorption and elution from calcium phosphate gel (15).

RESULTS AND DISCUSSION

Sialic acid levels in normal and subnormal semen

The sialic acid concentrations of 20 normal and 15 subnormal semen specimens were determined (Tables I and II). For this study the decision to place specimens into the normal and subnormal categories was based on laboratory investigation of the semen. Semen which contained less than 60,000,000 spermatozoa per ml. was considered subnormal (16, 17). Specimens with more than 60,000,000 spermatozoa per ml. with normal motility and sperm morphology were placed in the normal class.

The average number of spermatozoa was 139.7 million per ml. in normal and 19.3 million per ml. in the subnormal specimens. The mean volume of normal ejaculates was 3.6 ml., and 3.4 ml. in the subnormal. In two other studies the average ejaculate volumes of men from sterile marriages were 3.4 and 3.59 ml. (16, 18).

As seen in Table I the average sialic acid concentration of 20 normal semen specimens by the column method was 124.2 mg. per cent (standard error of mean, 8.7), with values ranging from 64.5 to 219.0 mg. per cent. Subnormal samples ranged from 42.4 to 134.9 mg. per cent with a mean of 90.1 mg. per cent (standard error of mean, 6.2), 27.5 per cent lower than the normal (Table II). The difference between the levels of sialic acid in normal and subnormal semen specimens is small but is statistically significant ($p < 0.01$). Svennerholm reported an average of 105.5

TABLE I

Sialic acid concentration in normal semen

Sample	Volume	Spermatozoa	Sialic acid
no.	*ml.*	*no./ml. × 10⁶*	*mg./100 ml.**
1	3.5	220	107.3
2	2.8	80	185.6
3	3.2	100	144.0
4	1.5	90	112.2
5	4.3	102	123.7
6	1.5	250	130.0
7	6.0	300	90.8
8	4.0	265	90.2
9	3.0	90	64.5
10	3.5	80	127.2
11	3.0	90	219.0
12	2.1	107	89.2
13	3.8	162	86.5
14	5.6	118	195.5
15	3.6	196	116.0
16	4.6	68	102.0
17	3.6	100	95.0
18	7.4	112	85.9
19	1.6	177	155.8
20	3.9	86	163.2
Mean	3.6	139.7	124.2
Standard error of mean			8.7

* To convert into millimole per liter, divide by 30.9.

TABLE II

Sialic acid concentration in subnormal semen

Sample	Volume	Spermatozoa	Sialic acid
no.	*ml.*	*no./ml. × 10⁶*	*mg./100 ml.*
1	1.0	8	103.8
2	3.0	20	104.2
3	4.0	15	103.8
4	1.0	1	62.8
5	2.2	27	42.4
6	2.3	0	134.9
7	2.0	7	63.9
8	4.8	14	101.5
9	3.8	35	109.7
10	3.6	5	94.0
11	7.2	11	117.7
12	3.0	38	75.7
13	4.4	43	61.2
14	3.4	29	80.5
15	5.4	37	95.6
Mean	3.4	19.3	90.1
Standard error of mean			6.2

mg. sialic acid per 100 Gm. of semen in four samples (2). The average amount of sialic acid in normal ejaculates was 4.39 mg. while in subnormal ejaculates it was 3.11 mg., 29.2 per cent less.

The sialic acid values listed above were obtained by the column method of Svennerholm. In Table III a comparison is made of sialic acid values obtained by the column assay and the Ehrlich assay carried out directly on semen. The Ehrlich assay values were higher due to a material in semen which was not sialic acid but which reacted with the Ehrlich reagent. This material was not adsorbed by the Dowex-1-acetate column and reacted only slightly in the orcinol and resorcinol tests. The differences between the results of the column and Ehrlich method were not constant from specimen to specimen. It is evident that the Ehrlich reaction must be used with caution in the assay of sialic acid in semen and probably in other biological materials as well. In this instance, a significant difference between sialic acid levels of normal and subnormal semen samples was masked in the Ehrlich assay.

Other colorimetric methods such as the orcinol, resorcinol, and diphenylamine assays may also be unsuitable for the measurement of sialic acid in tissues and secretions unless they are partially purified. Free sugars and other nonsialic acid materials in semen react in these tests and obscure the characteristic absorption spectra of the sialic acid chromophore. We have found that dialysis of semen results in a 35 to 85 per cent decrease in the peak optical densities in these reactions.

It may be seen from Tables I and II that a high sialic acid value is not necessarily associated with a high sperm count. The highest sialic acid value among the subnormal specimens was found in one which was devoid of spermatozoa. There is a wide variation in the seminal sialic acid concentration, as there is in the sperm count. This variation of sialic acid levels in semen is considerably greater than it is in serum (19) and synovial fluid (13), and may reflect not only the efficiency of synthesis but the frequency of ejaculation. Hotchkiss found in humans that the total sperm content of an ejaculation was 51 per cent less than one 24 hours previous (17). Sialic acid levels may decrease correspondingly. However, this factor has not been investigated.

Two of the major components of semen, prostatic and seminal vesicle secretions, markedly differ in their sialic acid concentrations (*vide infra*). Perhaps differences of the relative proportion of these secretions in semen in different individuals or in the same individual may be responsible for the wide variation in the sialic acid level.

TABLE III

Averages of sialic acid concentrations of semen by two methods

Method	Normal (20 samples)	Subnormal (15 samples)
	mg./100 ml.	*mg./100 ml.*
1. Column	124.2	90.1
2. Ehrlich reaction on semen	184.5	188.0

Sialic acid in human prostatic and seminal vesicle secretions

The presence of sialic acid in semen raises the question as to its site of synthesis. The bulk of semen comes from the prostate and seminal vesicles. Since the concentrations of sialic acid in prostatic and seminal vesicle fluids were not known, they were examined by the column method for their content of sialic acid.

It may be seen in Table IV that seminal vesicle fluid contains an average of 231.9 mg. of sialic acid per 100 ml. determined by the column method. To date, this is the highest sialic acid concentration found in any human tissue or fluid. The mean concentration of sialic acid in prostatic fluid is 60.6 mg. per cent(Table V), determined by the column method.

If these values are used it can be calculated that approximately two parts of prostatic fluid must be mixed with one part of seminal vesicle secretion to attain a concentration of 124.2 mg. per cent, the

TABLE IV

Sialic acid concentration of human seminal vesicle secretion

Sample	Sialic acid
no.	*mg./100 ml.*
1	216.0
2	230.2
3	212.0
4	269.5
Mean	231.9

TABLE V

Sialic acid concentration on normal human prostatic secretion

Sample	Sialic acid
no.	*mg./100 ml.*
1	56.8
2	84.9
3	39.6
4	71.8
5	55.1
6	36.9
7	75.8
8	63.0
Mean	60.6

average concentration in normal semen. In other words, the prostate may supply two-thirds the volume of the ejaculate. In two other studies, prostatic secretion has been estimated to comprise 41 to 63 per cent of semen (20, 21). However, Lundquist has reported that human semen consists of 15.4 to 32.5 per cent prostatic fluid, 46.3 to 80.4 per cent seminal vesicle fluid and 1.6 to 12.9 per cent testicular and epididymal secretion (22).

The testes, epididymus and ductus deferens add little to the volume (22, 23) and probably very little to the sialic acid content of semen. Although sialic acid has been isolated from testicular homogenates, spermatozoa which have been washed 12 times contain only a small amount of sialic acid (*vide infra*).

Sialic acid in spermatozoa

The removal of spermatozoa from semen by centrifugation does not significantly alter the sialic acid content of semen. There remains, however, the possibility that a small amount of sialic acid is present in the spermatozoa.

A pooled sample of normal semen, 10.5 ml. in volume, which contained 96.5 mg. per cent of sialic acid and 78×10^6 spermatozoa per ml. was centrifuged at $5{,}000 \times G$ for 10 minutes. The spermatozoa were washed 12 times at room temperature, twice with distilled water, six times with physiological saline, twice with 0.2 M acetate buffer, pH 5.0, and twice with 0.1 M potassium phosphate buffer, pH. 7.3. The spermatozoa were hydrolyzed and analyzed by the colmun method. The 1 M acetate eluate was tested by the Ehrlich and resorcinol tests and found to contain 0.054 mg. of sialic acid, or 0.53 per cent of the total sialic acid of the semen sample. Each sperm cell contains 2.1×10^{-10} μM of sialic acid.

It is unlikely that the spermatozoa are contaminated by mucoprotein after 12 washes. In three experiments, absorption spectra characteristic of sialic acid have been observed when the direct Ehrlich test was performed on washed spermatozoa. In one experiment it was observed that some of this sialic acid could be solubilized by partially purified neuraminidase. The function of sialic acid in spermatozoa, as in seminal mucoproteins, is unknown. Perhaps, in some manner sialic acid is involved in the union of ovum and spermatozoa as it is with the interaction of virus and red blood cell (7).

Dialyzable sialic acid in semen

Two ml. of semen was dialyzed against 100 ml. of distilled water, for 20 hours at 4° C. The dialysate was concentrated *in vacuo,* and then tested for sialic acid by the Ehrlich assay. In five experiments the amount of sialic acid found in the dialysate was 2.6, 3.7, 6.4, 4.0 and 4.8 per cent of the total seminal sialic acid value. Some of this material had the same R_f as N-acetylneuraminic acid in Solvent 1.

It is not known whether dialysable sialic acid actually exists in a free state, is bound to another sugar moiety, or is cleaved from seminal mucoprotein as the sample ages. This problem is being investigated at the present time.

The action of neuraminidase (sialidase) on semen

It was found that a maximum of 79 per cent of sialic acid was released from seminal mucoprotein by partially purified neuraminidase. The sialic acid released is probably bound to mucoprotein by the same neuraminidase sensitive linkage that binds sialic acid to orosomucoid (8).

Identification of sialic acid

Sialic acid was isolated from semen, seminal vesicle, testis and prostate as described in "Methods." These samples and authentic N-acetylneuraminic acid were adsorbed by Norit A and could be eluted with 50 per cent aqueous methanol.

TABLE VI

R_f values of sialic acid isolated from natural sources

Solvent	Sialic acid isolated from: Seminal vesicle	Prostate	Testes	Semen	N-Acetyl-neuraminic acid	N-Glycolyl-neuraminic acid	N,O-Diacetyl-neuraminic acid
1. *Sec*-butanol:acetic acid: water, 4:1:5	0.67	0.65	0.67	0.67	0.67		0.67
2. *n*-Butanol:acetic acid: water, 4:1:5	0.14	0.14	0.14	0.13	0.14		0.13
3. Ethyl acetate:pyridine: water, 1:1:2	0.69	0.70	0.70	0.68	0.70		0.70
4. *n*-Butanol:*n*-propanol: 0.1 *N* HCl, 1:2:1	0.42	0.41	0.41	0.41	0.42	0.33	0.41

All behaved similarly on columns of Dowex-1-formate and Dowex-1-acetate.

The R_f values of the materials from all four sources were the same as those of N-acetyl and N,O-diacetylneuraminic acid in four solvents (Table VI). In Solvent 4, the R_f of the isolated sialic acid differed from that of N-glycolylneuraminic acid (14). However N-acetylneuraminic acid and N,O-diacetylneuraminic acid have the same R_f values in all of the solvents used, and so it is not known from this data which of these forms is present.

To differentiate between N-acetyl and N,O-diacetylneuraminic acids, sialic acid was isolated from dialyzed human semen by a method which is used for the isolation of N,O-diacetylneuraminic acid from bovine submaxillary gland mucin (10). The seminal sialic acid was kept at 4° C. in 0.01 N H_2SO_4 for 18 hours, a process which is known to remove only the O-acetyl group from N,O-diacetylneuraminic acid (10). The solution was neutralized and enzymatically assayed for free acetate (24).[3]

In contrast to the sialic acid of bovine submaxillary gland mucin, no free acetate was detectable in the preparations of sialic acid from semen. This indicates that N-acetylneuraminic acid is the form that is present in human semen.

A solution of sialic acid, which was derived from semen, contained 0.23 mg. of sialic acid per ml. by the Ehrlich assay, 0.22 mg. per ml. by the orcinol, and 0.23 mg. per ml. by the resorcinol test. In each case N-acetylneuraminic acid served as the standard.

[3] Acetokinase was kindly supplied by Mr. W. E. Pricer, Jr.

SUMMARY

1. The average concentration of sialic acid in 20 normal human semen samples, by the anion exchange column method of Svennerholm, was found to be 124.2 mg. per 100 ml. with a range of 64.5 to 219.0 mg. per 100 ml. and a standard error of the mean of 8.7. The average concentration found in 15 human semen samples with a low content of spermatozoa was 90.1 mg. per cent with a standard error of the mean of 6.2 and a range of 42.4 to 134.9 mg. per cent. The difference between the sialic acid levels in normal and subnormal semen is small but statistically significant ($p < 0.01$). A small amount of the seminal sialic acid was found to be dialyzable. Partially purified neuraminidase removed a maximum of 79 per cent of the sialic acid from seminal mucoprotein.

2. The average sialic acid concentration of eight samples of human prostatic fluid was 60.6 mg. per cent. Four samples of human seminal vesicle secretion had an average sialic acid concentration of 231.4 mg. per cent. This is the highest concentration reported in any human tissue or secretion. It has been estimated from these values that the prostate accounts for approximately two-thirds the volume of semen while the seminal vesicles provide one-third.

3. In one experiment it was found that human spermatozoa contained 0.53 per cent of the total seminal sialic acid content.

4. Sialic acid, isolated from human semen, seminal vesicle, testis and prostate, has been compared to N-acetyl, N,O-diacetyl and N-glycolylneuraminic acid. N-Acetylneuraminic acid is the form of sialic acid found in human semen.

5. The column assay of Svennerholm, and the Ehrlich assay, are compared. The unreliability of colorimetric assays for sialic acid directly on semen is discussed.

ACKNOWLEDGMENTS

The author wishes to thank Mrs. M. L. Smith for technical assistance.

REFERENCES

1. Blix, F. G., Gottschalk, A., and Klenk, E. Proposed nomenclature in the field of neuraminic and sialic acids. Nature (Lond.) 1957, **179**, 1088.
2. Svennerholm, L. Quantitative estimation of sialic acids. III. An anion exchange resin method. Acta chem. scand. 1958, **12**, 547.
3. Chatagnon, C., and Chatagnon, P. L'acide neuraminique. Constituant naturel des tissus et des liquides biologiques de l'homme et des animaux; son interêt en biologie médicale. Presse méd. 1955, **63**, 1194.
4. Barry, G. T. Colominic acid, a polymer of N-acetylneuraminic acid. J. exp. Med. 1958, **107**, 507.
5. Cornforth, J. W., Firth, M. E., and Gottschalk, A. The synthesis of N-acetylneuraminic acid. Biochem. J. 1958, **68**, 57.
6. Comb, D. G., and Roseman, S. Composition and enzymatic synthesis of N-acetylneuraminic acid (sialic acid). J. Amer. chem. Soc. 1958, **80**, 497.
7. Gottschalk, A. Virus enzymes and virus templates. Physiol. Rev. 1957, **37**, 66.
8. Popenoe, E. A., and Drew, R. M. The action of an enzyme of Clostridium perfringens on orosomucoid. J. biol. Chem. 1957, **228**, 673.
9. Odin, L. Sialic acid in human cervical mucus, in hog seminal gel, and in ovomucin. Acta chem. scand. 1955, **9**, 1235.
10. Blix, G., Lindberg, E., Odin, L., and Werner, I. Studies on sialic acids. Acta Soc. Med. upsalien. 1956, **61**, 1.
11. Werner, I., and Odin, L. On the presence of sialic acid in certain glycoproteins and in gangliosides. Acta Soc. Med. upsalien. 1952, **57**, 230.
12. Svennerholm, L. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. Biochim. biophys. Acta 1957, **24**, 604.
13. Pigman, W., Hawkins, W. L., Blair, M. G., and Holley, H. L. Sialic acid in normal and arthritic human synovial fluids. Arth. and Rheum. 1958, **1**, 151.
14. Svennerholm, E., and Svennerholm, L. Quantitative paper partition chromatography of sialic acids. Nature (Lond.) 1958, **181**, 1154.
15. Warren, L. Unpublished data.
16. Johansson, C.-J. Clinical studies on sterile couples with special reference to the diagnosis, etiology and prognosis of infertility. Acta obstet. gynec. scand. 1957, **36**, Suppl. 5.
17. Hotchkiss, R. S. Fertility in Men. Philadelphia, J. B. Lippincott Company, 1944.
18. MacLeod, J. The male factor in fertility and infertility. An analysis of ejaculate volume in 800 fertile men and in 600 men in infertile marriage. Fertil. and Steril. 1950, **1**, 347.
19. Saifer, A., and Gerstenfeld, S. The serum neuraminic acid distribution. I. Methodology. J. Lab. clin. Med. 1957, **50**, 17.
20. Gutman, A. B., and Gutman, E. B. Quantitative relations of a prostatic component ("acid" phosphatase) of human seminal fluid. Endocrinology 1941, **28**, 115.
21. Hansen, P. F. Determination of the "acid" prostatic phosphatase as a new method for medicolegal demonstration of sperm spots. Acta path. microbiol. scand. 1946, **23**, 187.
22. Lundquist, F. Aspects of the biochemistry of human semen. Acta physiol. scand. 1949, **19**, Suppl. 66.
23. Mann, T. The Biochemistry of Semen. London, Methuen and Company, Ltd., 1954.
24. Rose, I. A. Acetate kinase of bacteria (acetokinase) *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. Academic Press Inc., New York, 1955, vol. I, p. 591.

TRANSMISSION OF THE COMMON COLD TO VOLUNTEERS UNDER CONTROLLED CONDITIONS. IV. SPECIFIC IMMUNITY TO THE COMMON COLD *

By GEORGE GEE JACKSON AND HARRY F. DOWLING

(From the Department of Medicine and the Research and Educational Hospitals, University of Illinois College of Medicine, Chicago, Ill.)

(Submitted for publication December 10, 1958; accepted January 5, 1959)

Although the common cold is the most prevalent illness of humans, information on the antigenicity of the infectious agents and the immune response of the host to the infection has been difficult to obtain. Epidemiologic investigations have led to divergent hypotheses. The frequent repetition of colds, on the one hand, has suggested little or no immunity to reinfection. On the other hand, the diminution or disappearance of colds among isolated populations, even in an arctic climate, is compatible with the belief that persons develop immunity to specific common cold agents (1, 2). Analysis of the influence of age upon susceptibility also is inconclusive with regard to acquired immunity to the common cold. The frequency of common colds tends to decline with age beyond childhood (3–5), but in a survey of colds within a group of families apparent evidence of brief immunity was observed after infection only among children (3).

Transmission of the common cold to volunteers by filtered nasal secretions or nasopharyngeal washings offers the most direct approach to the problem. Several groups of investigators have shown that the common cold can be successfully transmitted to volunteers, but almost always it has been impossible to infect all of them. The incidence of experimental colds has been about 35 to 50 per cent (6, 7). The basis for susceptibility or resistance is not known but might be specific immunity. In previous studies we have shown that the infectivity of filtered infectious nasal secretions can be neutralized by the globulin fraction, but not by albumin, from pooled human plasma (8). Andrewes has reported that serum of persons convalescent from the common cold has the capacity to inactivate the common cold virus (9). These data suggest that specific neutralizing antibody might in fact be developed during recovery from the common cold. In this paper, data are presented that show a significant degree of specific immunity among volunteers to a second challenge with the same infectious nasal secretion and a lack of immunity following challenge with a different nasal secretion.

* These studies were carried out under the sponsorship of the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, and were supported in part by the Office of the Surgeon General, Department of the Army, and in part by grants from the Lederle Laboratries Division, American Cyanamid Company, and from Charles Pfizer & Company, Inc.

MATERIALS AND METHODS

The details of the volunteer experiments have been published previously (7). Each nasal secretion that was given to volunteers was collected during the months from January through April from a different donor during a nonfebrile, coryzal illness which had the characteristics of a common cold. The secretions were stored in a refrigerator at −90° F. Later they were thawed and homogenized with an equal volume of Hanks salt solution. The sediment was separated by centrifugation. The supernatant was diluted until the final concentration of nasal secrtion was 1:5. The solution was then filtered through a sintered glass or porcelain (Selas 02) bacterial filter. The filtrate was diluted with an equal portion of a solution of stabilizing protein which was a five per cent solution of human type O hemoglobin until 1954, and since that time, a one per cent solution of yeast extract. Aliquots of the secretions were tested in a 1:2 or 1:10 dilution of the original secretion for recognizable viruses in embryonated eggs, suckling and adult mice and monolayers of Hela and monkey kidney cells in tissue culture. Paired sera from the donors were not always obtained but sera from volunteers given each agent were tested by complement fixation and/or neutralization tests for common respiratory viruses.

The volunteers were almost all students enrolled in nursing, medicine, dentistry or pharmacy. They had been free from symptoms of a spontaneous or experimental cold for at least six weeks. Subjects were placed in groups at the beginning of the experiment. The infectious challenge, which was instilled into each nostril of the recumbent subject, was 0.2 ml. of a 1:100 final dilution of nasal secretion or the control challenge consisting

TABLE I

Transmission of the common cold to volunteers

Challenge	Number challenged*	Experimental result: No cold	Cold	% colds
Nasal secretion	1,034	585	449	42
Buffer	696	626	70	10
Significance of difference	$\chi^2 = 377$, $p = <0.001$			

* Only the initial challenge with any one secretion is included.

of the same volume of buffered salt solution. The groups were kept isolated for 30 minutes before dismissal. They were requested to avoid close association with other volunteers and all persons with respiratory illness. Sneezing, headache, malaise, chilliness, sore throat, nasal discharge, nasal obstruction and cough were elicited from the subject and graded for the day prior to challenge and each day for six days after challenge by a person unaware of the nature of the nasal instillation. The increase in these symptoms over the amount present on the day of the challenge was totalled at the end of the week and designated the symptom score. The decision as to whether the subject had developed a cold was based upon three criteria: total symptom score, subjective recognition of a "cold" and the degree of increased nasal discharge (7).

RESULTS

The results of the initial challenge of 1,034 subjects given one of five infectious nasal secretions are given in Table I. On the basis of the criteria outlined above, 449, or 42 per cent, of them developed a cold. Among 696 control subjects simultaneously given buffered salt solution, only 70, or 10 per cent, developed a cold. The difference in the incidence of illness following the two types of inocula is highly significant.

TABLE II

Infectivity among volunteers of different nasal secretions from donors with a common cold

Nasal secretion: Year recovered	Code	Number challenged*	Experimental results: No cold	Cold	% colds
1953	69	219	136	83	38
1954	111	185	116	69	37
1955	127	261	164	97	37
1956	142	231	114	117	51
1957	154	138	55	83	60

* Only the initial challenge with any one secretion is included.

The five secretions were collected in successive years from 1953 to 1957. Each secretion was given to more than 100 volunteers. The infectivity of the individual secretions in the initial challenge of volunteers varied from 37 to 60 per cent as shown in Table II. For each secretion, the amount of illness transmitted was highly significant compared with control subjects.

The susceptibility on initial challenge of volunteers when they are arranged into groups according to whether the subject participated in one or several experiments and received the same or a different secretion is shown in Table III. Colds developed in 43 per cent of 660 persons given only one infectious challenge. Among 144 volunteers who were later rechallenged with the same secretion, 71, or 49 per cent, developed a cold from the initial challenge and 13, or 9 per cent, developed a cold from rechallenge; among 115 subjects subsequently given different secretions, 35 per cent had colds from the initial challenge and 43 per cent from the second secretion. Thus, natural host factors, if contributory, would appear to have

TABLE III

Comparison of the susceptibility of different groups of volunteers to the initial challenge with an infectious nasal secretion

Number of participations	Number challenged	Sequence of challenge	Experimental result: No cold	Cold	% colds
1 only	660	Initial	373	287	43
2 or more with same nasal secretion	144	Initial	73	71	49
	144	Rechallenge with same secretion	131	13	9)
2 or more with different nasal secretion	115	Initial	74	41	35
	115	Second nasal secretion	65	50	43)

TABLE IV

Response of volunteers to two successive challenges with the same infectious nasal secretion

Line	Result of experimental challenges: Initial challenge	Result of experimental challenges: Rechallenge	Number of volunteers who received: Nasal secretion no.: 69	111	127	142	154	Any nasal secretion	Buffer
1	No cold	No cold	2	3	24	16	21	66 (90%)	48
2	No cold	Cold	0	1	3	3	0	7 (10%)	2
3	Cold	No cold	0	6	14	17	28	65 (92%)	1
4	Cold	Cold	0	1	1	0	4	6 (8%)	0
	Total subjects challenged		2	11	42	36	53	144	51
	Colds developing after initial challenge (Lines 3 and 4)								
5		Number	0	7	15	17	32	71	1
6		Per cent	0	64	36	47	60	49	2
	Colds developing after rechallenge (Lines 2 and 4)								
7		Number	0	2	4	3	4	13	2
8		Per cent	0	18	10	8	8	9	4
9	Significance of difference Initial *vs.* rechallenge			<0.05	<0.01	<0.001	<0.001	<0.001	Not sig.

favored greater susceptibility in the group rechallenged with the same secretion.

The results of rechallenge with the same secretion are shown in Table IV. Among 73 volunteers (Lines 1 and 2) who had no cold following the initial challenge, 66, or 90 per cent, (Line 1) were again immune upon rechallenge. Among 71 others (Lines 3 and 4) who developed a cold from the initial challenge with one of the five secretions, 65, or 92 per cent (Line 3), were immune upon rechallenge with the same secretion. The mean interval between challenges was 15.2 weeks with a range of 3 to 45 weeks. The difference in susceptibility to the initial challenge and the subsequent rechallenge is highly significant ($p = < 0.001$), and since 10 per cent spontaneous colds were observed among a large number of subjects given a buffered salt solution (Table I) the results might represent complete immunity to rechallenge with the same agent.

A similar pattern of induced immunity was observed for each of the individual secretions. The per cent of colds after rechallenge (Table IV, Line 8) was always considerably less than after the initial challenge (Line 6). The difference was statistically significant (Line 9) beyond the chance level of 0.001 for the single Secretions 154 and 142, beyond the 0.01 level for Secretion 127 and the 0.05 level for Secretion 111.

A survey of the six subjects who developed a cold after both of two successive exposures to the same secretion (Table IV, Line 4) showed that in four of them the second of the two colds was definitely of lesser severity. The symptom score was only one-third to one-half that of the initial illness. The two remaining subjects developed symptoms on the first day of the experiment and had fever, neither of which were characteristic of the experimentally transmitted illness.

Among the seven volunteers who developed a cold only after the second of two challenges with the same secretion (Table IV, Line 2), the time of onset and character of the illness indicated that two of them almost surely had a cold which originated outside of the experiment and five appeared to have been genuinely susceptible and developed a cold from the second challenge only. The interval between challenges for those subjects who had a cold upon rechallenge (Lines 2 and 4) was 7 to 34 weeks, statistically the same as for the entire group.

The data were analyzed to determine whether the decreased infectivity observed upon rechallenge might be an artifact owing to diminished

TABLE V

The simultaneous infectivity of stored nasal secretions in different types of volunteers participating in the same experiments

Nasal secretion	Volunteers receiving initial challenge		Volunteers receiving a rechallenge		Significance of difference
	No.	% colds	No.	% colds	Initial *vs.* rechallenge
111	53	30	11	18	Not sig.
127	96	42	35	11	<0.001
142	89	47	25	12	<0.001
154	90	60	33	6	<0.001
Sum	314	48	104	10	<0.001

infectivity of the secretions upon storage. That this was not the case is shown by the comparison of infectivity among subjects given an initial challenge simultaneously with those who were rechallenged (Table V). The number of observations is smaller than the total number who were rechallenged since all of the experiments did not contain both types of subjects. The differences in infectivity of the secretion between initial and rechallenged volunteers in the same experiments are highly significant for each of the secretions for which 25 to 35 observations were available for comparison.

Evidence regarding the specificity of the different secretions and the similarity of the volunteers except for their acquired specific immunity was obtained by comparing the results among subjects rechallenged with the same secretion and those who were later challenged with a different secretion. The comparison is shown in Figure 1. Volunteers were observed to be immune to rechallenge with the same secretion, shown by the left hand pair of bars in the lower two panels, regardless of the clinical result of the initial challenge, shown in the upper panel. This was a significantly different result than was observed among volunteers who were rechallenged with a different secretion illustrated by the right hand pair of bars in the lower two panels. The latter subjects were just as susceptible to the second challenge as the general population, as observed from the results among persons who participated in a single experiment.

Susceptibility to the second secretion also was independent of whether the initial challenge caused a cold or no cold. Among 74 subjects who were

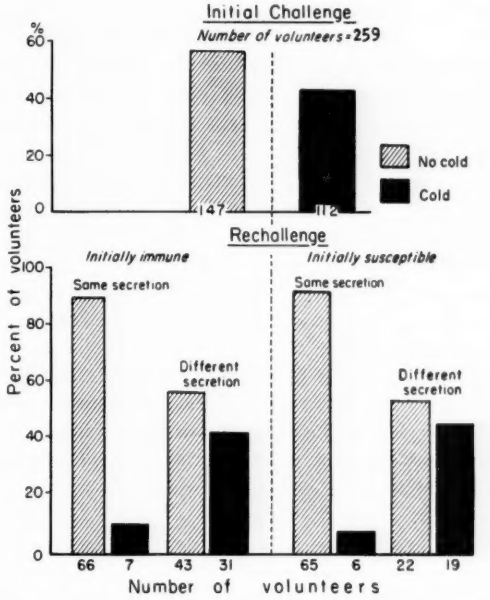


FIG. 1. RESPONSE OF VOLUNTEERS TO TWO CHALLENGES WITH INFECTIOUS NASAL SECRETION

initially immune to one secretion and were later challenged with a different secretion, 31, or 40 per cent, developed a cold. Similarly, among 41 initially susceptible volunteers who had a cold after the first challenge and were subsequently challenged with a different secretion, 19, or 46 per cent, developed a cold. These results are statistically the same and are also nearly identical to the results of the initial challenge of the same subjects. They are significantly different from the results of rechallenge with the same secretion among either the initially immune or initially susceptible volunteers.

The sequence of administration of different secretions was examined for evidence of cross-immunity among persons given more than one infectious secretion. The data are shown in Table VI. The mean interval between challenges was 30.6 weeks with a range of 10 to 74 weeks. No interrelationship is apparent. The order in which the different secretions were used was dependent upon their time of recovery, and thus all possible sequences were not examined. Overall, colds were observed in 43 per cent of volunteers after the latter of two agents given in sequence, and in

TABLE VI

Lack of acquired cross-immunity to common cold nasal secretions

Sequential challenge				Single challenge only			Significance of difference
Prior challenge	Later challenge						
Secretion	Secretion	Number challenged	% colds	Secretion	Number challenged	% colds	Sequence *vs.* single challenge
69	111	15	20	111	108	37	None
69 or 111	127	61	31	127	127	39	None
69, 111 or 127	142	36	72	142	158	46	<0.05
127 or 142	154	3	67	154	84	58	None
Total		115	43		477	45	None

45 per cent among others who were challenged only with the latter agent. Thus, in contrast to the specific protection to rechallenge conferred by any one secretion, no cross-protection was demonstrated among the different secretions.

DISCUSSION

The data are quite conclusive evidence for the development of specific immunity to a second cold from the same infectious nasal secretion under the conditions of the experiments. They imply that the five agents from secretions collected in successive years are immunologically distinct and that one-third to two-thirds of the volunteer population was, initially, specifically immune to each of the agents. Previously, analysis of the incubation period had suggested that some of the agents were different from one another (*7*). These findings raise many questions as to how they are related to common experience with respiratory infections, previous epidemiologic and clinical investigations, and finally as to the identity and number of common cold viruses.

The first critique should consider the validity of the methods by which the data were obtained. In any infection the response of the primary host to an infectious challenge is considered the ultimate test for immunity. Volunteer studies satisfy this requirement. The basic protocols for the replicate experiments were similar, so that all subjects were challenged in the same manner and with the same volume and dilution of the filtered nasal secretion (*7*). The differences were not caused by season, nonspecific characteristics of the volunteers, nor a change in the secretions upon storage. We have not used any pooled secretions nor repassaged agents.

The common cold is principally an afebrile, subjective, coryzal illness (*7*). There is therefore some margin of error in the proper designation of volunteers with an experimental cold. In these experiments, we adhered rigidly to criteria previously outlined as delineating an experimental cold (*7*). Also, the experiments were controlled by the inclusion of randomly-selected volunteers who received only buffered saline. The statistical differences observed greatly exceeded any error from the experimental design. Finally, since the volunteer population included 1,730 subjects in approximately 90 separate experiments performed over a period of more than five years, variations in individual experiments were minimized.

One phase of the experiments that was not ideal is the lack of isolation of the subjects before and during the experiments. Some of the illnesses observed were possibly from community contacts, and the community exposure might have produced colds or immunity that would make the data impure. Such "wild" colds would have tended to give a falsely high proportion of colds. The significant finding in these experiments was a low incidence of colds among particular groups of volunteers. The observed immunity pertained to only one secretion and was therefore specific whether it was acquired experimentally or from outside contacts. The high degree of statistical significance in the results, when analyzed either by simultaneous comparison of initial volunteers with those who were being rechallenged, or by longitudinal comparison of volunteers who were rechallenged with those who participated only

once and with uninfected control subjects, strongly suggests that concomitant community infections were unimportant in the interpretation of the results.

The duration of the specific immunity cannot be estimated beyond the 3 to 45 weeks observed. Thus, immunity might not last from one season of respiratory diseases to another; on the other hand, if protection from reinfection is based upon the development of specific antibody, immunity might last much longer.

With regard to prior epidemiologic and investigative data, the principle of specific immunity would be an adequate basis upon which to explain the findings in the classic observations in Greenland (1) and at Spitsbergen (2) and the historical report of acute upper respiratory diseases among inhabitants of the arctic island of St. Kilda (10). Specific antibody is the presumed basis for the neutralization of the infectious secretion by human globulin (8) and by serum from persons convalescent from a common cold (9).

In the detailed studies of respiratory illnesses among Cleveland families, if secondary attack rates were taken as an index of the relative immune status of the individual, the descending order of immunity was observed to be fathers, mothers and older school children, school children aged less than 6 years and, finally, preschool children. The findings of Lidwell and Sommerville (3) and Buck (11) were essentially the same. The latter investigator found some indication of decreased susceptibility to colds among preschool children and among adults during the two months following a cold but not among infants or among children aged 5 to 14 years.

Likewise, Reid, Williams and Hirch (12) noted some indication of short-term immunity during an epidemic of colds among office workers.

Badger and his associates (5, 13) believe that immunity develops as a result of the aging process rather than from repeated experience with the agent and cite as evidence the fact that the incidence of colds was not reduced in children as a result of attendance at school during the previous year. Furthermore, preschool children living with older school children may be assumed to have had excessive exposure in the recent past but, in spite of this, secondary attack rates for preschool children are similar regardless of whether or not there are school children in the family.

These objections to the development of immunity following colds might be explained by the presence, either simultaneously or in succession, of many antigenically-different viruses capable of producing the common cold.

Observations of the Commission on Acute Respiratory Diseases (14) are more difficult to explain. These investigators rechallenged five volunteers after 19 days with filtrate from a subject with a "severe common cold" and produced colds in four in the initial challenge and four in the rechallenge. Three of the latter four had had a cold on the first challenge.

The explanation of these results, which are contradictory to ours, is not known. It is possible, however, that the dose of nasal washings used was important. In each subject they used 5 ml. which was atomized and nebulized into the nose and throat (15). Even so, these investigators stated that, in general, those who had experienced a previous illness as a result of inoculation with the same filtrate suffered from a somewhat milder infection upon reinoculation.

Observations confirmatory to ours were made in chimpanzees by Dochez, Shibley and Mills (16), who stated that these animals could only rarely be infected with secretions from patients with the common cold when challenged within three months after having a previous infection.

The nasal secretion (NS) agents we have reported upon have all failed to grow in animals and in tissue cultures and are believed not to be among the presently known viruses.

Serologic studies of selected pairs of sera from volunteers have shown no relationship of the experimental infections to adeno-viruses, influenza A, B or C, Sendai virus, hemadsorption viruses, chimpanzee coryzal agent (CCA), croup-associated virus (CA), J.H. and 2060 viruses, or polio virus types 1, 2 or 3. These studies are being extended to help clarify the role of the known viruses in the common cold. The identity of the NS agents is being sought and their characterization must ultimately be worked out. At the present time, volunteer studies are essential and can contribute significantly in our understanding of the common viral infections of the respiratory tract. The data

presented would suggest that the pathogenesis and host response to the common cold are comparable to those that occur in many other specific viral infections.

SUMMARY AND CONCLUSIONS

The frequency with which the common cold occurred in volunteer subjects who received one of five infectious secretions obtained from patients with common colds or a control buffer solution was as follows:

1. Among 1,034 subjects who received an infectious secretion as an initial challenge, 449, or 42 per cent, developed colds as compared with 70, or 10 per cent, of 696 subjects who served as a control population and simultaneously received a buffer solution.

2. Among 73 subjects who developed no cold on initial challenge, 7, or 10 per cent, developed colds on rechallenge with the same infectious secretion. Among 71 subjects who developed a cold on initial challenge, 6, or 8 per cent, developed a cold on rechallenge. The interval between challenges varied from 3 to 45 weeks. There was no evidence that the secretions had lost their infectivity between challenges.

3. In contrast, among 74 subjects who developed no cold on initial challenge with an infectious secretion, 31, or 40 per cent, developed a cold when challenged later with another secretion. Among 41 subjects who developed a cold on initial challenge with an infectious secretion, 19, or 46 per cent, developed a cold when challenged later with another secretion. The interval between challenges varied from 10 to 74 weeks.

4. It is concluded that the instillation of one of the five infectious secretions obtained from patients with a common cold gave specific protection against the development of a common cold when the same secretion was instilled up to 45 weeks later, whereas there was no protection against a cold produced by any other secretion. These observations suggest that the common cold is caused by a number of different viruses which are antigenically different, and that following infection human subjects develop a specific immunity against the virus which lasts at least for several months.

ACKNOWLEDGMENTS

We wish to thank the following persons for assistance in this study: Truman O. Anderson, Ph.D.; Arthur V. Boand, Ph.D.; Kenneth Hovland; Tohru Inouye, M.S.; Louise R. Jensen; Jack Saporta; Delores H. Seketa; the late Irwin G. Spiesman, M.D.; Marvin Turck; and Frieda K. Webb.

REFERENCES

1. Heinbecker, P., and Irvine-Jones, E. I. M. Susceptibility of Eskimos to the common cold and a study of their natural immunity to diphtheria, scarlet fever and bacterial filtrates. J. Immunol. 1928, **15**, 395.
2. Paul, J. H., and Freese, H. L. An epidemiological and bacteriological study of the common cold in an isolated arctic community (Spitsbergen). Amer. J. Hyg. 1933, **17**, 517.
3. Lidwell, O. M., and Sommerville, T. Observations on the incidence and distribution of the common cold in a rural community during 1948 and 1949. J. Hyg. (Lond.) 1951, **49**, 365.
4. Downes, J. Change with age in susceptibility to minor respiratory illness. Milbank mem. Fd Quart. 1952, **30**, 211.
5. Badger, G. F., Dingle, J. H., Feller, A. E., Hodges, R. G., Jordan, W. S., Jr., and Rammelkamp, C. H., Jr. A study of illness in a group of Cleveland families. II. Incidence of the common respiratory diseases. Amer. J. Hyg. 1953, **58**, 31.
6. Andrewes, C. H. The common cold. Brit. med. Bull. 1953, **9**, 206.
7. Jackson, G. G., Dowling, H. F., Spiesman, I. G., and Boand, A. V. Transmission of the common cold to volunteers under controlled conditions. I. The common cold as a clinical entity. A.M.A. Arch. intern. Med. 1958, **101**, 267.
8. Jackson, G. G., Dowling, H. F., and Anderson, T. O. Neutralization of common cold agents in volunteers by pooled human globulin. Science 1958, **128**, 27.
9. Andrewes, C. H. Adventures among viruses. III. The puzzle of the common cold. New Engl. J. Med. 1950, **242**, 235.
10. Gafafer, W. M. The inhabitants of the island of St. Kilda and acute upper respiratory disease. Hum. Biol. 1931, **3**, 437.
11. Buck, C. Acute upper respiratory infections in families. Amer. J. Hyg. 1956, **63**, 1.
12. Reid, D. D., Williams, R. E. O., and Hirch, A. Colds among office workers; epidemiological study. Lancet 1953, **2**, 1303.
13. Badger, G. F., Dingle, J. H., Feller, A. E., Hodges, R. G., Jordan, W. S., Jr., and Rammelkamp, C. H., Jr. A study of illness in a group of Cleveland families. IV. The spread of respiratory infections within the home. Amer. J. Hyg. 1953, **58**, 174.

14. Commission on Acute Respiratory Diseases. Experimental transmission of minor respiratory illness to human volunteers by filter-passing agents. II. Immunity on reinoculation with agents from the two types of minor respiratory illness and from primary atypical pneumonia. J. clin. Invest. 1947, **26**, 974.

15. Commission on Acute Respiratory Diseases. Experimental transmission of minor respiratory illness to human volunteers by filter-passing agents. I. Demonstration of two types of illness characterized by long and short incubation periods and different clinical features. J. clin. Invest. 1947, **26**, 957.

16. Dochez, A. R., Shibley, G. S., and Mills, K. C. Studies in the common cold. IV. Experimental transmission of the common cold to anthropoid apes and human beings by means of a filtrable agent. J. exp. Med. 1930, **52**, 701.

THE RELATION OF URINARY CO_2 TENSION TO BICARBONATE EXCRETION *

By RICHARD M. PORTWOOD,†‡ DONALD W. SELDIN, FLOYD C. RECTOR, JR. AND ROBERT CADE

(From the Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas, Texas)

(Submitted for publication October 23, 1958; accepted January 22, 1959)

The mechanism whereby high CO_2 tensions are formed in the urine has been the subject of considerable debate. Pitts and Alexander (1) proposed that the CO_2 tension in alkaline urines is increased as a result of the following sequence: *1)* increased delivery of $NaHCO_3$ to the distal tubule; *2)* increased formation of H_2CO_3 as a result of a Na^+-H^+ exchange in this segment; *3)* delayed dehydration of the H_2CO_3 thus formed. More recently, Kennedy, Eden and Berliner (2) have emphasized the role of nonbicarbonate buffer as the factor responsible for delayed dehydration of H_2CO_3. It is apparent, therefore, that variations in the excretion of buffer as well as bicarbonate might influence the urinary CO_2 tension.

The high CO_2 tensions that follow the administration of Diamox® are particularly difficult to explain within this conceptual framework, because Diamox® undeniably suppresses overall renal H^+ secretion. Evidence has recently been presented (3) to support the thesis that the high CO_2 tension in urine during a bicarbonate diuresis induced by Diamox® is the consequence, not of the enhanced bicarbonate excretion, but of the buffering action of the inhibitor.

The present studies were designed to examine in man the relationship of buffer and bicarbonate excretion to urine CO_2 tension by observing the pattern of various types of bicarbonate diuresis, including that produced by Diamox®, with and without the simultaneous infusion of phosphate buffer.

* This work was supported in part by a grant from the National Institutes of Health, United States Public Health Service, and in part by a grant from the Dallas Heart Association.

† This work was performed while a Public Health Service Trainee of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

‡ Present address: Division of Endocrinology, School of Medicine, Duke University, Durham, N. C.

MATERIALS AND METHODS

A total of 27 experiments were performed on normal young adults (medical students, house officers and student nurses) after an overnight fast.

Maximum water diuresis was maintained by the rapid intravenous infusion of either 5 per cent dextrose or 5 per cent fructose in water. The subjects remained recumbent throughout the experiments except for those individuals from whom voided urine specimens were obtained. Urine collection periods of 15 to 20 minutes were employed; the samples were collected under mineral oil by direct voiding in some experiments, by indwelling catheter in most. At the mid-point of each urine collection period blood was drawn into heparinized, oiled syringes from an indwelling needle in either a brachial or femoral artery.

Blood and urine pH were anaerobically determined immediately with a Cambridge pH meter with the electrode assembly housed in an incubator at a constant temperature of 37° C. Plasma and urine CO_2 content were determined by the method of Van Slyke and Neill (4). Bicarbonate concentration and pCO_2 were calculated from the Henderson-Hasselbalch equation. The pK_1 of H_2CO_3 in urine was estimated for each sample according to the formula: $pK_1 = 6.33 - 0.5\ \sqrt{B}$, where B represents the total cation concentration of the sample in question (5). It was assumed that the sum $[Na^+]+[K^+]$ was equal to at least 95 per cent of $[B^+]$. It was found that the arbitrary selection of a pK_1 of 6.10 introduced a variable error of considerable magnitude when calculating pCO_2 in urines of varying pH and bicarbonate concentration. However, using the pK_1 estimated from $6.33 - 0.5\ \sqrt{B}$, close agreement was obtained between calculated pCO_2 and tonometrically determined pCO_2. The conventional proportionality constant of 0.0309 was used to convert $[H_2CO_3]$ to pCO_2. For plasma, a pK_1 of 6.10 and a proportionality constant of 0.0301 were used.

Inorganic phosphate in urine was determined by the method of Fiske and Subbarow (6). Titration curves for urines obtained during several of the experiments were determined as follows:

Ten ml. urine was acidified to $<$ pH 2.0 by the addition of 0.1 HCl. Following vigorous stirring for 15 minutes (to dispel generated CO_2), the samples were titrated to pH 6.0 to 6.2 with 0.1 N NaOH. Further titration to pH $>$ 8.0 was performed with 0.01 N NaOH which was

TABLE I

The relation of U−P pCO_2 to bicarbonate excretion during maximal water diuresis

Subject, experiment	Time	Plasma			Urine						
		pH	HCO_3^-	pCO_2	pCO_2	U−P pCO_2	pH	HCO_3^-		$PO_4^{=}$		Flow
	min.		*mEq./L.*	*mm. Hg*	*mm. Hg*	*mm. Hg*		*mEq./L.*	*μEq./min.*	*mM/L.*	*μM/min.*	*ml./min.*
C. G.	0– 15	7.35	23.9	44	57	13	6.22	1.8	53	0.5	15	29.3
	16– 35*	7.34	23.1	44	47	3	6.36	2.1	95	0.2	9	45.0
Diamox®	36– 55	7.35	23.1	43	64	21	7.11	16.1	383	0.2	5	23.8
	56– 75	7.34	23.1	44	66	22	7.17	19.2	662	0.6	21	34.5
	76– 95	7.36	22.9	42	67	25	7.08	15.6	468	0.5	15	30.0
	96–115	7.33	22.8	44	77	33	6.98	14.3	422	0.7	21	29.5
J. H.	– 40	Begin infusion 7.5% $NaHCO_3$ at 6 ml./min.										
	0– 20	7.41	27.4	45	62	17	7.23	20.6	268	1.9	25	13.0
Metabolic	21– 40	7.42	30.8	49	78	29	7.38	36.3	490	1.5	20	13.5
alkalosis†	41– 60	7.45	31.5	47	89	42	7.45	49.0	564	1.7	20	11.5
	61– 80	7.52	32.8	42	88	46	7.49	53.1	770	1.6	23	14.5
	81–100	7.45	35.2	52	90	38	7.55	62.6	864	1.5	21	13.8
J. W.	0– 20	7.39	23.4	39	44	5	5.81	0.6	14			21.5
	21– 40‡	7.38	24.0	40	41	1	6.01	0.9	24			25.0
Respiratory	41– 60	7.50	19.3	26	28	2	6.35	1.3	38			29.0
alkalosis	61– 80	7.52	19.0	25	24	−1	6.60	2.0	52			26.0
	81–100‡	7.49	17.7	24	22	−2	6.58	1.8	40			22.5
	101–120	7.39	22.0	37	36	−1	6.56	2.5	72			29.0
	121–140	7.37	23.6	39	31	−8	6.50	2.0	44			22.0
C. D.	0– 20	7.41	24.9	40	56	16	6.28	2.1	66			31.5
	21– 40	200 ml. 7.5% $NaHCO_3$ I.V.										
Metabolic	41– 60*	7.47	36.3	51	97	46	7.49	65.9	1,865			28.3
alkalosis§	61– 80	7.50	36.4	48	68	20	7.65	65.9	2,069			31.4
+	81–100	7.49	32.5	46	66	20	7.63	61.8	1,452			23.5
Diamox®	101–120	7.50	34.7	46	70	24	7.62	64.1	1,699			26.5
J. W.	0– 24	7.40	24.7	41	39	−2	6.01	0.9	19			20.4
	25– 53*	7.41	25.6	42	45	3	5.83	0.7	13			18.3
Respiratory	54– 76‡	7.37	23.6	42	62	20	7.17	18.2	546			30.0
alkalosis	77– 98	7.51	17.1	22	48	26	7.34	20.8	449			21.6
+	99–118	7.48	18.2	25	47	22	7.39	22.6	593			26.2
Diamox®	119–139‡	7.54	15.5	19	45	26	7.34	18.4	648			35.2
	140–161	7.38	19.8	34	47	13	7.16	13.4	341			25.4
	162–181	7.35	20.4	38	52	14	6.98	9.8	290			29.5
N. K.	0– 15	7.38	23.5	41	52	11	6.33	2.2	60	0.1	3	27.3
	16– 30*	7.39	23.6	40	59	19	6.27	2.1	47			22.3
Respiratory	31– 45‖	7.39	23.7	40	65	25	7.16	18.3	512	0.05	1	28.0
acidosis	46– 60	7.27	24.8	56	83	27	7.20	25.8	697	0.3	8	27.0
+	61– 75‖	7.23	24.0	59	94	35	7.07	21.5	688	0.4	13	32.0
Diamox®	76– 90	7.34	21.5	41	76	35	7.16	21.4	456	0.7	15	21.3
	91–105	7.41	22.6	37	94	57	7.26	33.5	425	1.0	13	12.7
J. H.	0– 20‖	7.40	23.2	39	48	9	6.32	2.0	38			19.0
	21– 40	7.32	25.1	50	60	10	6.35	2.6	49			19.0
Respiratory	41– 55	7.27	25.1	56	67	11	6.23	2.2	47			21.3
acidosis	56– 70‖	7.23	25.7	63	68	5	6.19	2.1	43			20.7
	71– 85	7.35	24.2	45	66	21	6.44	3.6	54			15.3

* Injection of 250 mg. Diamox® intravenously.
† The infusion of 7.5 per cent $NaHCO_3$ was given at the rate of 6 ml. per minute for 40 minutes before urines were collected and continued throughout the experiment at this rate.
‡ Period of hyperventilation.
§ The infusion of 7.5 per cent $NaHCO_3$ was given at the rate of 10 ml. per minute for 19 minutes, after which the rate was slowed to 5 ml. per minute.
‖ Period of breathing 6 per cent CO_2.

added in small increments with pH determination following each addition.

Eight types of experiments, as charted below, were performed to examine the relationship between bicarbonate excretion and the urine minus plasma (U−P) pCO_2 gradient under conditions of varying plasma acid-base composition and varying bicarbonate and buffer excretion.

Type

Respiratory alkalosis
Respiratory alkalosis + Diamox®
Respiratory acidosis
Respiratory acidosis + Diamox®
Metabolic alkalosis
Metabolic alkalosis + Diamox®
Diamox® only
Diamox® + phosphate infusion
Metabolic alkalosis + phosphate infusion

Respiratory alkalosis was induced by voluntary hyperventilation, assisted by a Haliburton IPPB machine, respiratory acidosis by breathing 6 to 6.5 per cent CO_2 in oxygen. Metabolic alkalosis was produced by the intravenous infusion of hypertonic (7.5 per cent) $NaHCO_3$ at rates of 2 to 6 ml. per minute. The phosphate solutions used were made from Na_2HPO_4–NaH_2PO_4 (pH 7.4), diluted to isotonicity and delivered at rates of 4 to 8 ml. per minute. Diamox® was given intravenously as the sodium salt in a single injection of 250 mg.[1]

RESULTS

Representative experiments are charted in Table I. Urine pCO_2 was increased following the in-

[1] The authors wish to thank Lederle Laboratories Division, American Cyanamid Company, for generously supplying the Diamox® used in these experiments.

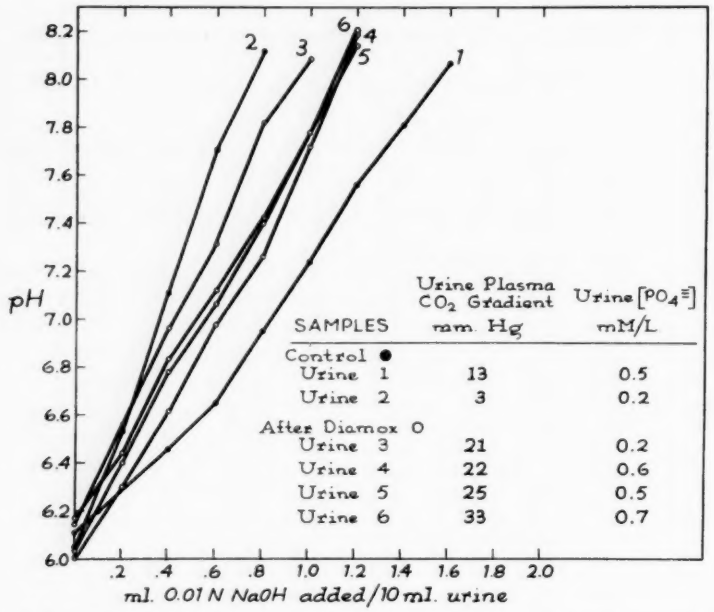


FIG. 1. TITRATION CURVES OF URINES OBTAINED BEFORE AND AFTER DIAMOX® ADMINISTRATION DURING MAXIMAL WATER DIURESIS

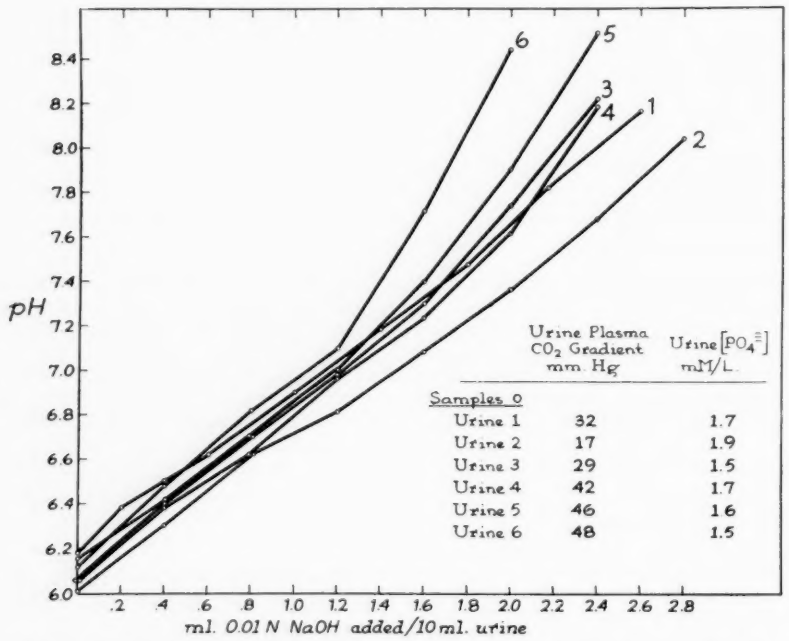


FIG. 2. TITRATION CURVES OF URINES OBTAINED DURING BICARBONATE DIURESIS AND MAXIMAL WATER DIURESIS

TABLE II

Effects of massive buffer excretion on U−P pCO_2 during bicarbonate diuresis

Subject, experiment	Time	Plasma			Urine							
		pH	HCO_3^-	pCO_2	pCO_2	U−P pCO_2	pH	HCO_3^-		$PO_4^{=}$		Flow
	min.		*mEq./L.*	*mm. Hg*	*mm. Hg*	*mm. Hg*		*mEq./L.*	*μEq./min.*	*mM/L.*	*μM/min.*	*ml./min.*
L. J.	0– 20	7.39	21.1	36	42	6	6.22	1.3	22	0.6	10	17.0
	21– 40*	7.37	21.1	38	50	12	6.08	1.2	17	0.6	8	14.0
Diamox®	41– 60	7.36	20.9	38	56	18	6.98	10.4	222	0.8	17	21.3
only	61– 80	7.36	19.1	35	63	28	7.27	22.9	573	1.0	25	25.0
	81–100	7.38	19.2	33	64	21	7.27	23.1	462	1.0	20	20.0
	101–120		20.7	35	63	28	7.24	21.5	441	1.0	20	20.5
L. J.	– 40	Start isotonic buffer phosphate at 8 ml./min.										
Diamox®	0– 20	7.36	19.7	36	37	1	5.88	0.6	7			11.0
+	21– 40*	7.34	20.0	38	36	−2	5.75	0.4	5	17.6	229	13.0
massive	41– 60	7.35	20.1	38	96	58	6.84	12.9	271	16.6	349	21.0
PO_4	61– 80	7.34	20.0	38	74	36	7.06	16.7	276	19.5	322	16.5
infusion†	81–100	7.36	20.0	37	82	45	7.00	16.1	282	20.7	362	17.5
	101–120	7.34	20.1	39	77	38	7.04	16.3	274	18.1	304	16.8

* Injection of 250 mg. Diamox® intravenously.
† Isotonic sodium phosphate (pH 7.4) was infused at the rate of 8 ml. per minute for 40 minutes before the start of urine collections and was continued at this rate throughout the experiment.

jection of Diamox® (C.G.), during $NaHCO_3$ infusion (J.H.), during $NaHCO_3$ infusion and Diamox® administration (C.D.), during respiratory acidosis with Diamox® (N.K.) and during respiratory acidosis alone (J.H.). It was decreased during respiratory alkalosis alone (J.W.) and was not appreciably increased during respiratory alkalosis plus Diamox® (J.W.).

These changes in the absolute value of urine pCO_2 cannot automatically be interpreted to indicate increased generation of CO_2 from H_2CO_3 formed within the tubular lumen. Since CO_2 diffuses freely across cell membranes, alterations in plasma pCO_2 will change the pCO_2 in urine without any associated change in intraluminal H_2CO_3 production. To correct for this, pCO_2 has been expressed as the difference between urine and plasma CO_2 tension (7). The ratio U/P pCO_2 (2) has not been used because the same U−P gradient might result in different ratios. In the last period of Experiment C.G., U−P was 33 mm. and U/P was 1.75. In the last hyperventilation period of J.W., U−P was 26 mm., but a higher U/P ratio of 2.37 was observed.

It is clear from Table I that appreciable bicarbonate diuresis, no matter how induced, was invariably associated with a definite increase in U−P gradient. The urine concentration of phosphate was exceedingly low throughout the studies and did not increase appreciably during the experiments in which phosphate was not infused.

To determine whether enhanced excretion of buffers other than phosphate might account for the high U−P gradients during $NaHCO_3$ diuresis, titration curves were determined from urines obtained following Diamox® administration (Figure 1) and $NaHCO_3$ infusion (Figure 2). Despite the fact that a very dilute solution of NaOH was used to titrate the urines, no increases in buffer concentration between control and experimental samples could be detected. Nevertheless, marked increases in U−P pCO_2 occurred. Similar curves were obtained in all experiments where bicarbonate diuresis was produced without phosphate loads.

That great increases in buffer excretion do, in fact, enhance U−P is seen in the representative experiments listed in Table II, where phosphate excretion was increased 20-fold by a phosphate infusion administered during a Diamox® induced diuresis. A similar effect of phosphate was found in other studies where $NaHCO_3$ and phosphate were infused.

Figure 3 illustrates the relation between bicarbonate excretion and U−P pCO_2 for all studies. Under the conditions of these experiments, where maximal water diuresis was maintained, a mean gradient of about 30 mm. Hg was obtained at bicarbonate excretion of approximately 300 μEq. per minute, irrespective of the manner in which bicarbonate diuresis was produced. Increasing bicarbonate excretion beyond this point did not increase U−P.

DISCUSSION

An increase in the CO_2 tension of the urine may result from several different processes. First, ele-

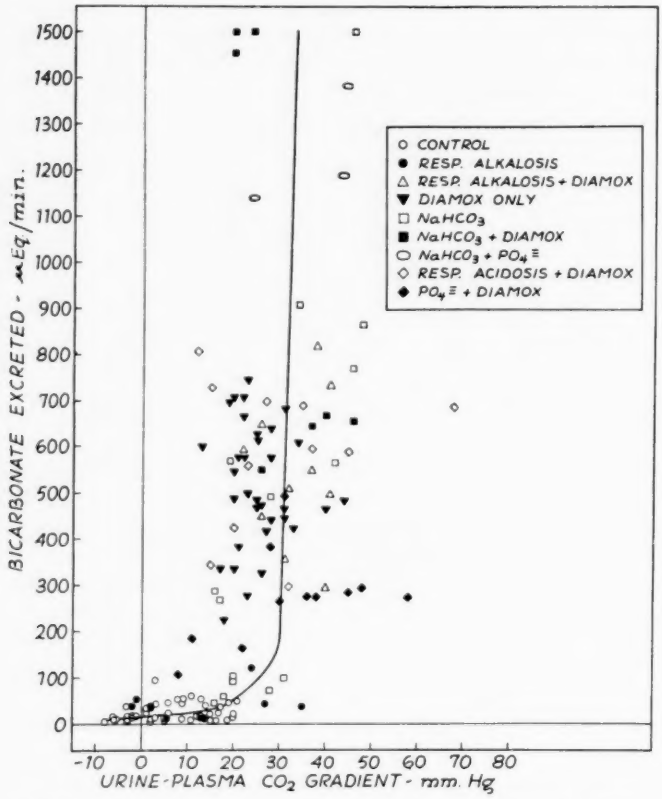


FIG. 3. RELATIONSHIP BETWEEN HCO_3^- EXCRETION AND URINE MINUS PLASMA CO_2 GRADIENT DURING MAXIMAL WATER DIURESIS

vations of plasma pCO_2 during respiratory acidosis would increase urinary CO_2 tension because of the rapid diffusion of CO_2 between renal blood and tubular urine. Second, the mixture in the bladder of urines of alkaline and acid pH, such as occurs during prolonged urine collection periods when plasma acid-base composition is changing, would readily generate high CO_2 tensions. Third, H_2CO_3 might be generated in the collecting ducts as a result of the admixture of acid and alkaline urines originating in heterogeneous nephrons (7). Finally, the secretion of H^+ by distal tubular cells into luminal fluid containing HCO_3^- would also result in the formation of H_2CO_3 (1). It should be emphasized that, in order for these last two processes to elevate the U−P gradient, it is necessary that the dehydration of H_2CO_3 be delayed. The obliteration of U−P gradients in alkaline urines following the injection of carbonic anhydrase in amounts sufficient to cause its excretion into the urine (8) strongly supports the central role attributed to delayed dehydration, although it does not establish which of the last two processes is responsible for the formation of H_2CO_3.

The effects of changes in plasma CO_2 tension are obviated by employing the urine minus plasma (U−P) gradient of pCO_2 rather than the absolute value of pCO_2 as a basis for comparison. The elevation of plasma CO_2 tension during metabolic alkalosis and respiratory acidosis, although raising the CO_2 tension of the urine, would have no effect on the gradient. In the present studies, the short urine collection periods, maximal water diuresis, and constant experimental circumstances all combine to minimize the effects of bladder mixing. The increased U−P gradient observed in all ex-

periments where noteworthy bicarbonate diuresis occurred is, therefore, attributable to events within the kidney.

Kennedy, Orloff and Berliner (*7*) have proposed that acid and alkaline urines delivered from heterogeneous nephrons are mixed in the collecting duct system, thereby forming H_2CO_3 and hence resulting in a high U−P gradient. In addition to the admixture of acid and alkaline urines, the mixing hypothesis requires, as Kennedy and co-workers point out, that the acid urine involved contain some buffer in order to generate increased urinary CO_2 tension. Inasmuch as the urines obtained in the present studies contained so little buffer, it seems unlikely, although not impossible, that admixture of heterogeneous urines could account for the observed gradients.

Under the conditions of the studies, the high U−P gradients are most reasonably attributed to accelerated delivery of $NaHCO_3$ to the distal tubule, where the reabsorption of Na^+ in exchange for H^+ generated large amounts of H_2CO_3. The dehydration of H_2CO_3 thus formed was delayed, owing to the absence of carbonic anhydrase from tubular urine. Complete dehydration of H_2CO_3, therefore, occurs lower in the urinary tract where unfavorable volume-surface relationships, or less permeable epithelium, serves to perpetuate the gradient.

While accepting the thesis that delayed dehydration of H_2CO_3 is responsible for the high U−P gradients in alkaline urines, Kennedy, Eden and Berliner (*2*) have presented evidence from *in vitro* studies to show that the dehydration of H_2CO_3 is immeasurably rapid in the absence of nonbicarbonate buffer. They therefore propose that in order for dehydration of H_2CO_3 to be delayed, buffer must be present in the tubular urine. It follows from this that in alkaline urines, the U−P gradient might rise as a result of increased buffer excretion independently of changes in the magnitude of bicarbonate diuresis.

In the present study increased U−P gradients following $NaHCO_3$ infusions were not accompanied by changes in buffer excretion. Moreover, owing to the fasting state and the maximal water diuresis, the concentration of urinary buffer was exceedingly low. It appears, therefore, that very small amounts of buffer will delay the dehydration of H_2CO_3 sufficiently to generate high U−P gradients. The fact that massive phosphate excretion resulted in a still further increment in U−P gradient suggests that the excretion of buffer, although influencing urine CO_2 tension to some extent, has only a minor effect in the range of buffer excretion ordinarily encountered.

It is especially noteworthy that Diamox® increased the U−P gradient to about the same extent as infusions of $NaHCO_3$ (Figure 3). This is more difficult to explain, inasmuch as the inhibition of renal carbonic anhydrase is associated with diminished overall H^+ secretion. It has recently been suggested that Diamox® elevates the U−P gradient by its buffering action rather than by its effect on carbonic anhydrase (*3*). If sufficient Diamox® is given, as appears to have been the case in the experiments just cited, its buffering action may be sufficient to produce these effects. This hypothesis, however, could hardly account for the present results. The amounts of Diamox® given were comparatively small (less than 1 mM), phosphate excretion did not appreciably increase, and titration curves disclosed no detectable rise in excretion of total urinary buffer.

Despite the fact that Diamox® depresses overall H^+ secretion, it is likely that accelerated production of H_2CO_3 accounts for the high U−P gradients. If the major effect of Diamox® were on the proximal tubules with only minor inhibition exerted distally, then the increased amounts of $NaHCO_3$ which escaped proximal tubular reabsorption would be delivered to the distal tubular exchange site. Here, the Na^+-H^+ exchange process would generate large amounts of H_2CO_3 even though the maximum capacity of the distal tubular system to secrete H^+ might be somewhat depressed. In keeping with this explanation is the recent evidence suggesting that a major site of action of Diamox® is the proximal tubule (*9*).

If buffer excretion does not vary greatly, the magnitude of the U−P gradient appears to be determined by the rate of distal H^+ secretion relative to the rate of Na^+ reabsorption, as $NaHCO_3$ is delivered to the exchange site. From Figure 3, it is apparent that a maximum gradient of approximately 30 mm. Hg is established during water diuresis at a HCO_3^- excretion rate of about 300 μEq. per minute. This represents the formation of at least 30×0.0309 or approximately 1 mM per L. H_2CO_3 in excess of that generated in

urines with no gradient. In addition, of course, some H_2CO_3 doubtless decomposes and is reabsorbed, so that the total distal H^+ secretion would be much higher.

It is conceivable that Diamox® might generate high gradients by inhibiting red cell carbonic anhydrase. Inhibition of CO_2 hydration in the red cell might elevate the CO_2 tension of renal plasma and interstitial fluid. Urine equilibrating with these fluids would therefore have a high CO_2 tension. This elevation of pCO_2 would not be reflected in systemic blood since sufficient time would elapse in the passage of the blood to the heart and lungs to permit the hydration of CO_2 via the uncatalyzed reaction to proceed to equilibrium. Thus, the calculation of the U−P gradient using the CO_2 tension of arterial blood would give a falsely high value. Against such a mechanism, however, is the fact that renal blood flow is very high in relation to the rate of CO_2 production by the kidney, so that inhibition of red cell carbonic anhydrase should have little effect on renal plasma pCO_2. Moreover, the relation between U−P and HCO_3^- excretion following the administration of Diamox® (Figure 3) is similar to that following $NaHCO_3$ infusions, suggesting that the generation of H_2CO_3 in the distal tubule is responsible for the U−P gradients in both instances.

SUMMARY

1. The relationship between bicarbonate diuresis and urine minus plasma (U−P) pCO_2 gradient was examined in man during maximum H_2O diuresis.

2. It was found that during water diuresis, a maximum U−P pCO_2 of about 30 mm. Hg developed at a HCO_3^- excretion rate of about 300 μEq. per minute. Buffer excretion was minimal during most of the experiments. The source of the H_2CO_3 (subsequently dehydrated to CO_2 and H_2O) is most logically attributed to the secretion of H^+ into luminal fluid containing HCO_3^-.

3. Marked increases in urine buffer excretion following phosphate infusions increased U−P gradients moderately, supporting the hypothesis that buffer can influence urinary CO_2 tension by delaying the dehydration of H_2CO_3.

4. The high U−P gradients generated during Diamox® diuresis appear to be the consequence of Na^+-H^+ exchange as $NaHCO_3$ floods the distal tubule, even though total H^+ secretion at the distal tubular exchange site may be somewhat depressed. This suggests that the principal site of action of Diamox® is the proximal tubule.

REFERENCES

1. Pitts, R. F., and Alexander, R. S. The nature of the renal tubular mechanism for acidifying the urine. Amer. J. Physiol. 1945, **144**, 239.
2. Kennedy, T. J., Jr., Eden, M., and Berliner, R. W. Interpretation of urine CO_2 tension. Fed. Proc. 1957, **16**, 72.
3. Capeci, N. E., Kruesi, O. R., Weaver, D. C., and Hilton, J. G. Effect of Diamox® (acetazoleamide) on the carbon dioxide tension of the urine. Amer. J. Physiol. 1957, **191**, 55.
4. Van Slyke, D. D., and Neill, J. M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. J. biol. Chem. 1924, **61**, 523.
5. Hastings, A. B., and Sendroy, J., Jr. The effect of variation in ionic strength on the apparent first and second dissociation constants of carbonic acid. J. biol. Chem. 1925, **65**, 445.
6. Fiske, C. H., and Subbarow, Y. The colorimetric determination of phosphorus. J. biol. Chem. 1925, **66**, 375.
7. Kennedy, T. J., Jr., Orloff, J., and Berliner, R. W. Significance of carbon dioxide tension in urine. Amer. J. Physiol. 1952, **169**, 596.
8. Ochwadt, B. K., and Pitts, R. F. Effects of intravenous infusion of carbonic anhydrase on carbon dioxide tension of alkaline urine. Amer. J. Physiol. 1956, **185**, 426.
9. Pitts, R. F. Some reflections on the mechanism of action of diuretics. Amer. J. Med. 1958, **24**, 745.

A PRACTICAL STUDY OF THE AIR CHAMBER MODEL OF THE CARDIOVASCULAR SYSTEM

By SIDNEY ROSTON and LEONARD LEIGHT

(From the Department of Medicine, University of Louisville School of Medicine, Louisville, Ky.)

(Submitted for publication September 9, 1958; accepted January 5, 1959)

Use of the air chamber as a simplified model of the cardiovascular system was studied for many years (1, 2). Subsequently, interest in this model declined, since it failed to yield strikingly useful results. Unfortunately, other methods for describing the behavior of the cardiovascular system in terms of such easily measurable quantities as arterial pressure and pulse rate also were unsuccessful (3, 4). The recent development by one of the authors of a more exact mathematical approach to the air chamber spotlighted the strengths and weaknesses of this model anew (5). Its chief strengths were its great simplicity and the fact that its theoretical behavior agreed with a considerable body of cardiovascular physiology such as the general shape of the arterial pressure curve (5). Its weakness, from the practical viewpoint, was that its application required the use of parameters which could not be determined by direct measurement in the body. The present study of the air chamber model had two objectives. In the first place, an attempt was made to evaluate the closeness of mathematically predicted results to measured data. Then, since the mathematical approach appeared to have validity, the practical usefulness of the model itself was evaluated.

EXPERIMENTAL

The source of experimental data was the human cardiac catheterizations performed at the Louisville General Hospital during the past three years. Only patients with cardiovascular abnormalities requiring further investigation were subjected to cardiac catheterization. Not used in the present study were records incomplete either because of technical difficulties or for other reasons, such as the fact that the cardiac output was not determined in patients below the age of 13 years. In addition, a few catheterizations were omitted because of auricular fibrillation. All other 59 records of the three year period were included, regardless of postcatheterization diagnosis. Previously described techniques of cardiac catheterization were used (6). Data adequate for this study included the output from the left ventricle into the aorta, the average arterial pressure and the arterial pressure tracing.

The quantities necessary for a mathematical description of the chamber model were as follows:

- t = time
- $P(t)$ = pressure in the aorta near the aortic valve. In the present study, this was approximated by the brachial artery pressure (7)
- $V(t)$ = volume equivalent to the aorta
- $F(t)$ = effective rate of peripheral blood flow
- P_D = end-diastolic pressure
- P_S = maximum systolic pressure
- $PP = P_S - P_D$ = pulse pressure
- $K = \Delta P/\Delta V$ = elasticity of the aorta
- $R = P(t)/F(t)$ = effective peripheral resistance
- i = seconds between the onset of systole and the occurrence of P_S
- P_i = decrease in pressure in the i seconds after the onset of diastole
- CO = number of liters of blood passing the aortic valve per minute
- P_{av} = average aortic pressure
- a = duration of the cardiac cycle in seconds.

The values which were determined by use of the tracing of the brachial artery pressure included P_D, P_S, PP, i, P_i, a, and the ratio, $-K/R$ ($= G$) (Figure 1). P_i and G were determined from plots of the natural logarithms of the diastolic pressures in the tracings *vs.* time. The onset of a rapid drop in aortic pressure was arbitrarily taken as the beginning of diastole. Ordinarily, the time of this rapid drop could be determined within 0.02 second without difficulty. In nearly every case, a straight line represented the relationship between ln $P(t)$ and t during diastole very closely (Figure 2). The value of G was the slope of this line. P_i was also determined from this line, since the initial portion of the actual pressure tracing during diastole

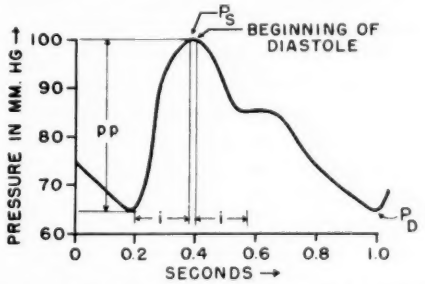


FIG. 1. A PATTERN OF BRACHIAL ARTERY PRESSURE
For this curve, a equals 0.8 second.

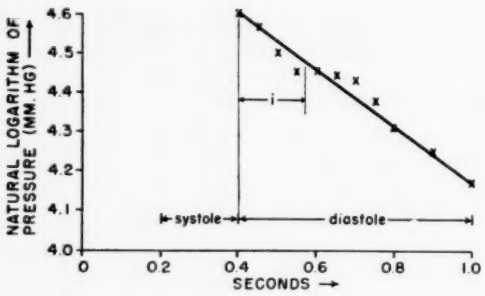


FIG. 2. A PLOT OF THE NATURAL LOGARITHM OF P *vs.* T FOR THE DIASTOLIC PRESSURE OF FIGURE 1

P_i equals 12 mm. Hg; $-K/R$ equals 0.72 second^{-1}.

was distorted somewhat from the theoretically anticipated logarithmic decline as a result of the closure of the aortic valve. In each arterial pressure tracing, three consecutive, nearly identical cycles were averaged. Quantities which were independently measured included P_{av} and CO.

Only one equation which could be tested in a practical way was derived from the mathematical formulation. This was

$$P_{av} = \frac{PP + P_i}{aG}. \qquad 1)$$

All of the quantities on the right hand side of Equation *1* were determined from the arterial pressure tracing alone. The value of P_{av} calculated from Equation *1* was then compared with the measured value, and the closeness of the two values provided an indication of the overall reasonableness of the theory. R was determined from the relationship, $R = \frac{60\ P_{av}}{CO}$, and substitution of this value of R into $-K/R = G$ yielded K.

RESULTS

The results of the 59 catheterizations were compiled in Table I. The ages of the patients ranged from 14 to 58 years, the average age being 31.5, with a standard deviation of 13 years. There were only six normal catheterization studies. Diagnoses included aortic insufficiency, mitral stenosis, mitral insufficiency, patent ductus arteriosus, peripheral arteriovenous fistula, anomolous pulmonary vein, interauricular septal defect and coarctation of the aorta. The wide range of each of the measured parameters was consistent with the great variety of pathology present.

On the average, the calculated value of P_{av} ($= P_{av-c}$) was 1.07 ± 0.07 times the measured value ($= P_{av-m}$), while, if P_i were omitted, P_{av-c} became 0.86 ± 0.09 times P_{av-m}. In each case, the number after the $\pm$ sign was the standard deviation. For the six patients with normal catheterization findings, P_{av-c} was 1.04 ± 0.07 times P_{av-m}. On the average, P_i was 25 ± 8 per cent of the corresponding pulse pressure. With use of the directly measured value of P_{av}, the mean value of R was $1,620 \pm 760$ dyne-seconds per cm.^{5}, and the average value of K was $1,470 \pm 770$ dynes per cm.^{5}, while the average value of K/R was 0.92 ± 0.33 seconds^{-1}. When the calculated value of P_{av} was used, the mean value of both R and K increased by 7 per cent, a change not statistically significant.

DISCUSSION

In the present study, P_{av-c} was nearly equal to P_{av-m} for a wide variety of cardiovascular pathology, as well as for normal hearts, over a range of ages from 14 to 58 years. The closeness of the theoretical and measured values of P_{av} indicated the practical feasibility of the air chamber model, insofar as this could be tested at present. The demonstration of the general validity of the theoretical approach was of considerably greater importance than was mere development of another method for the determination of P_{av}. From an empirical point of view, indeed, P_{av} might be more closely and easily approximated by such relationships as the sum of the diastolic pressure and one-third the pulse pressure than by Equation *1*. The significance of Equation *1*, however, lay in the fact that it was derived from application of the mathematical theory to the chamber model and could have no other origin, pragmatic or theoretical.

Some of the small deviation of P_{av-c} from P_{av-m} was due to errors in the measurements alone, such as those from the use of the Fick principle, and to the fact that all of the measurements were not recorded simultaneously. In addition, the brachial artery pressure differed from the pressure at the root of the aorta, among the differences being a slightly earlier occurrence and slightly greater magnitude of the peak of systolic pressure in the brachial artery than in the aorta (7). The term P_i brought the calculated value of P_{av} considerably closer to the observed value and decreased the deviation of the ratio, P_{av-c}/P_{av-m}, from its average value. For individuals undergoing exercise, with rapid heart rate, vigorous heart action and rapid initial decline of diastolic pressure, the relative

TABLE I

Results of 59 cardiac catheterization studies *

Case no.	Initials	Age	Sex	Diagnosis	P_S mm. Hg	PP mm. Hg	P_{av-m} mm. Hg	CO	m	a sec.	R† dyne-sec./cm.^{5}	K† dyne/cm.^{5}	$\frac{P_i}{PP} \times 100$	$\frac{P_{av-c}}{P_{av-m}} \times 100$
1	C. M.	14	M	Valvular pulmonic stenosis	112	52	75	1.86	7	0.65	3,220	3,870	17	105
2	E. W.	14	M	Coarctation of aorta	149	57	117	7.62	4	0.67	1,230	980	23	112
3	J. B.	15	M	Infundibular pulmonic stenosis	140	60	100	4.75	6	0.70	1,680	1,680	23	106
4	C. T.	15	F	Patent ductus arteriosus	116	47	94	7.31 (L→R 2.29)	4	0.56	1,030	1,160	26	99
5	H. M.	15	M	Patent ductus arteriosus	116	56	80	4.85 (L→R 1.55)	4	0.60	1,310	1,880	38	112
6	M. M.	15	F	Acute idiopathic pericarditis Normal catheterization	121	51	92	6.40	6	0.73	1,150	970	20	108
7	R. M.	17	M	Coarctation of aorta-aortic insufficiency	190	116	130	7.07	6	0.84	1,470	1,860	17	98
8	H. G.	17	M	Normal	130	60	90	7.79	7	0.65	920	1,050	15	103
9	G. S.	17	F	Rheumatic heart disease, mitral stenosis	84	27	65	2.80	5	0.98	1,850	1,000	30	102
10	R. P.	18	M	Patent ductus arteriosus	105	45	80	9.90 (L→R 4.15)	6	0.60	650	760	24	99
11	B. G.	18	F	Pulmonary hypertension of undetermined etiology	134	54	96	6.19	4	0.57	1,240	1,490	30	106
12	C. M.	18	F	Patent ductus arteriosus	98	34	76	2.51 (L→R 0.46)	4	0.96	2,410	1,210	21	112
13	J. M.	19	M	Pulmonic stenosis	109	33	85	7.28	5	0.95	930	400	18	112
14	M. C.	20	F	Normal	94	36	70	3.26	5	0.81	1,720	1,240	22	108
15	A. P.	21	F	Normal	123	70	80	8.10	4	0.80	790	1,030	31	110
16	B. C.	22	F	Rheumatic heart disease, mitral stenosis	90	28	74	4.11	3	0.83	1,440	760	36	117
17	S. H.	22	M	Normal	106	36	94	2.93	5	0.62	2,560	2,300	31	90
18	W. J.	22	F	Rheumatic heart disease, mitral stenosis	99	33	77	4.24	8	0.76	1,450	870	6	100
19	M. W.	23	F	? Interatrial septal defect	97	33	80	3.94	7	0.78	1,620	960	15	103
20	J. W.	23	F	Rheumatic heart disease, mitral stenosis	102	32	78	2.95	6	0.68	2,110	1,370	19	110
21	P. M.	23	F	Coarctation of aorta	230	110	160	11.2	5	0.69	1,150	1,410	24	100
22	B. T.	25	F	Anomalous pulmonary vein	111	41	88	3.55	5	0.80	1,960	1,350	22	104
23	A. M.	25	M	Valvular pulmonic stenosis	126	46	90	5.59	6	0.86	1,280	860	22	108
24	B. H.	25	F	Rheumatic heart disease, mitral stenosis	124	50	85	4.83	4	0.74	1,410	1,270	34	118
25	J. W.	26	M	Rheumatic heart disease, mitral stenosis	158	69	110	3.57	7	0.76	2,460	2,070	13	111

* Abbreviations are as follows: P_S, maximum systolic pressure; P_D, end-diastolic pressure; PP ($= P_S - P_D$), pulse pressure; P_{av-m}, average aortic pressure, measured value; CO, number of liters of blood passing aortic valve per minute; m, integer nearest to the actual value of the ratio, $\frac{a}{\text{duration of systole in seconds}}$; a, duration of cardiac cycle in seconds; R, effective peripheral resistance; K, elasticity of the aorta; P_i, decrease in pressure in the i seconds after the onset of diastole; i, seconds between the onset of systole and the occurrence of P_S; P_{av-c}, average aortic pressure, calculated value.

† K and R were determined with the use of P_{av-m}.

TABLE I—*Continued*

Case	Initials	Age	Sex	Diagnosis	Ps	PP	P_{av-m}	CO	m	a	R†	K†	$\frac{P_1}{PP} \times 100$	$\frac{P_{av-c}}{P_{av-m}} \times 100$
no.					*mm. Hg*	*mm. Hg*	*mm. Hg*			*sec.*	*dyne-sec./cm.⁵*	*dyne/cm.⁵*		
26	W. W.	26	M	? Interatrial septal defect	100	45	80	5.73	5	0.79	1,110	890	20	107
27	R. M.	26	F	Rheumatic heart disease, mitral insufficiency	96	36	74	2.75	3	0.69	2,140	1,710	31	115
28	H. T.	26	M	Coarctation of aorta, aortic insufficiency	192	108	117	11.40	5	0.64	820	1,310	24	112
29	C. B.	27	F	Rheumatic heart disease, mitral stenosis	100	30	82	4.20	4	0.56	1,560	1,450	47	103
30	H. J.	27	F	Normal	124	54	88	4.85	7	0.67	1,460	1,460	13	103
31	B. M.	29	M	Rheumatic heart disease, aortic stenosis and insufficiency, mitral stenosis	140	88	90	4.98	4	0.78	1,440	2,380	26	96
32	R. P.	30	F	Pulmonary arteriovenous fistula	110	57	72	9.30	4	0.74	620	720	32	121
33	A. M.	30	F	Rheumatic heart disease, mitral stenosis, post commisurotomy	105	20	92	4.30	3	0.68	1,710	790	30	90
34	A. M.	30	F	Rheumatic heart disease, mitral stenosis, pre-commisurotomy	105	42	83	2.30	4	0.69	2,880	3,180	43	95
35	J. C.	35	M	Rheumatic heart disease, mitral stenosis	93	28	72	4.00	5	0.54	1,440	1,050	21	120
36	N. S.	36	F	Patent ductus arteriosus	130	60	100	6.45	4	0.46	1,240	2,260	40	100
37	L. T.	38	M	Arteriosclerotic heart disease, with left heart failure	118	33	95	1.91	6	0.55	3,970	2,780	18	107
38	L. T.	39	F	Anomalous pulmonary vein	109	42	90	3.14	4	0.69	2,290	2,020	26	97
39	G. S.	40	M	Coarctation of aorta	148	68	100	4.91	5	0.73	1,630	1,650	28	118
40	J. B.	40	M	Hypertensive cardiovascular disease, with left heart failure	168	62	129	5.20	4	0.68	1,980	1,690	26	104
41	J. R.	41	M	Rheumatic heart disease, mitral stenosis	100	30	80	7.71	4	0.65	830	620	37	105
42	G. C.	41	F	Pulmonary hypertension of undetermined etiology	143	48	110	10.80	3	0.77	810	540	35	115
43	G. T.	41	M	Arteriosclerotic heart disease, with right and left ventricular failure	130	53	105	4.15	5	0.73	2,020	1,720	23	100
44	E. O.	42	M	Arteriosclerotic heart disease, with right and left ventricular failure	121	46	88	3.94	5	0.76	1,780	1,250	15	113
45	T. T.	43	M	Traumatic arteriovenous fistula, right thigh	128	60	85	10.9	5	0.97	740	560	28	123
46	G. D.	43	F	Arteriosclerotic heart disease, with left ventricular failure	119	57	82	5.41	4	0.72	1,210	1,410	33	111
47	W. H.	43	M	Pulmonary hypertension of undetermined etiology	138	60	98	11.38	5	0.74	690	690	28	106
48	T. T.	43	M	Arteriovenous fistula, right thigh (80 sec. after fistula closed by pressure)	157	70	108	4.15	6	1.30	2,060	1,110	20	111
49	M. J.	46	F	Normal	120	68	80	5.90	5	0.72	1,080	1,480	25	108
50	H. C.	47	M	Rheumatic heart disease, mitral stenosis, pre-commisurotomy	107	50	75	3.76	8	1.43	1,590	910	12	91

TABLE I—*Continued*

Case	Initials	Age	Sex	Diagnosis	P_s	PP	P_{av-m}	CO	m	a	R†	K†	$\frac{P_i}{PP} \times 100$	$\frac{P_{av-e}}{P_{av-m}} \times 100$
no.					*mm. Hg*	*mm. Hg*	*mm. Hg*			*sec.*	*dyne-sec./cm.*^{5}	*dyne/cm.*^{5}		
51	H. C.	47	M	Rheumatic heart disease, mitral stenosis, post-commisurotomy	115	48	82	5.31	5	0.88	1,230	990	27	105
52	F. S.	49	M	Rheumatic heart disease, mitral stenosis	99	32	80	5.20	4	0.80	1,230	770	31	105
53	M. M.	49	F	Rheumatic heart disease, mitral insufficiency	137	53	110	4.30	4	0.51	2,040	2,450	26	99
54	G. C.	50	F	Pulmonary hypertension of undetermined etiology	185	100	125	3.99	4	0.87	2,500	2,910	34	105
55	G. W.	52	M	Rheumatic heart disease, mitral stenosis	108	40	82	3.65	5	0.94	1,800	920	13	114
56	H. W.	54	M	Arteriosclerotic heart disease, aortic insufficiency, cor pulmonale	160	102	90	3.66	5	0.72	1,960	3,410	27	115
57	O. B.	54	M	Rheumatic heart disease, mitral stenosis	134	68	92	3.95	4	0.86	1,860	2,010	31	104
58	E. C.	58	F	Interatrial septal defect	154	64	108	4.50 (L→R 6.40)	5	0.60	1,920	2,070	23	113
59	H. K.	58	M	Rheumatic heart disease, mitral stenosis	126	46	90	2.12	4	0.79	3,390	2,440	33	119

value of P_i might be considerably greater than for the resting catheterization subjects.

With this demonstration of the validity of the mathematical model, it appeared reasonable to pursue the practical implications further. Attempts to determine the cardiac output from the arterial pressure tracing alone were unsuccessful. The cardiac output could not be determined from the pressure tracing until the value of K or R was known. However, while the ratio K/R could be derived from the pressure tracing, the determination of individual values of K and R required use of the measured cardiac output. Thus, an insoluble situation existed.

The value of the resistance, R, could be directly derived from measurements of P_{av} and CO. The distribution of R in the present study was broad, with the standard deviation nearly equal to one-half the mean value. Only minimal correlation of R with the diagnosis existed. Thus, for example, the average value of R for the eight patients with patent ductus arteriosus, peripheral arteriovenous fistula or aortic insufficiency, which might be expected to be low, actually was $1,350 \pm 560$, quite close to the overall mean value. On the other hand, the highest values of R occurred in patients with no obvious aortic or peripheral pathology. Age, sex and body size had no consistent effect upon R. Its value was less a function of gross anatomical abnormalities than of the state of constriction of the arterioles throughout the body. Furthermore, the wide variation of R in different individuals in essentially identical physical surroundings indicated that R was of more value as an index of emotional than of physical adjustment to an environment. R would probably be more useful in evaluation of the circulatory changes within an individual in different circumstances than in comparison of one individual with another.

In contrast to the resistance, which could be calculated from readily measurable quantities, the direct determination of K would be impossible in the living body. This direct determination required measurement of changes of aortic pressure as a function of nonperiodic changes in volume. Hence, during life, K would have to be determined by indirect methods. The distribution of K in the present study was broad, with a standard deviation greater than one-half the mean value. K

TABLE II

Variation of the aortic elasticity with age

Age	No. in group	Average K	Standard deviation	Standard deviation of mean
yrs.				
10–19	13	1,410	820	228
20–29	18	1,390	420	99
30–39	7	1,830	920	346
40–49	15	1,170	540	140
50–58	6	2,290	780	318

showed no consistent change with age up to 58 years (Table II). The results in Table II were in remarkably close agreement with values found in direct measurement of the pressure-volume relationship in the aortas of post-traumatic human cadavers (8). The 47 postmortem aortas yielded an average change of 16 ± 6.5 ml. in aortic volume per M.^{2} of body surface as an accompaniment of an increase in pressure from 80 to 110 mm. Hg. Since 1.78 was the mean value of the body surface area of the cadavers, K was equal, on the average, to 1,400 dyne per cm.^{5}—a mean value only 5 per cent less than, and statistically identical to, the average value of 1,470 (standard deviation of mean = 100) found in the present study, but distinctly different ($p < 0.01$) from the mean value of $1,023 \pm 482$ (standard deviation of mean = 58) found by Shock (9). The similarity of the values of K found in the work of Remington, Noback, Hamilton and Gold (8) and in the present study strongly suggested that the volume of the chamber in the mathematical model was essentially equivalent to the volume of the aorta. The deviation of the pressure in the distal aorta from the idealized pressure in the model as a result of the standing wave effect (10) apparently did not decrease the validity of the theoretical representation of the aorta by the chamber. In addition, Remington and his group showed that, up to approximately 115 mm. Hg, the value of K for aortas of individuals younger than 58 years showed no consistent pattern. However, above 58 years, the value of K increased. The similarity between the results found in cadavers and the results of the present study probably arose from the fact that the elasticity of the aorta, residing mainly in the inert elastic fibers, did not depend upon the viability of the body. The significance of K lay in its possible correlation with such factors as the degree of arteriosclerosis of the aorta.

The air chamber model gave evidence in the present study of present and potential usefulness. Its use led to an indirect method for the determination of K. With a known value of D, K and R could be determined by means of the arterial pressure tracing without measurement of P_{av}. If a measured value of D were unavailable, D could be roughly approximated from the arterial pressure tracing by assumption of a value for K. Furthermore, additional work would indicate whether or not the value of K remained reasonably constant in the same individual under various physiological circumstances. If it did remain reasonably constant, relative changes in R and D, though not the absolute values of these parameters, could be determined in a given individual from the arterial pressure tracing alone. Finally, the arterial pressure tracing might, under certain circumstances, be replaced by measurement of the blood pressure at the arm with a cuff. The auscultatory blood pressure would approximate the actual values of P_S and P_D in the brachial artery. From the pulse rate, the value of a could be determined, and tables of the average duration of diastole as a function of cardiac rate could be applied. An approximation to the value of K/R could be calculated from $\frac{\ln (P_S/P_D)}{\text{duration of diastole}}$. From this linear relationship, too, P_1 could be easily approximated. Thus, approximate determination of relative changes in R and D in a given individual might, under certain conditions, be feasible merely from measurement of the blood pressure at the arm with a cuff.

SUMMARY

The recent development of a more exact mathematical approach to the air chamber model of the cardiovascular system suggested evaluation of the practical validity of the mathematical representation and, more importantly, of the usefulness of the model itself. The source of experimental data was 59 cardiac catheterizations of adults with cardiovascular abnormalities. The mathematical approach proved valid insofar as it could be tested, the 7 per cent difference between the average arterial pressure directly measured and that derived

by means of the theory from the arterial pressure tracing being within the range of reasonable experimental error. The usefulness of the air chamber was indicated by the similarity of the aortic elasticities determined from the model to values previously found in direct measurements upon the aortas of cadavers. This similarity opened new possibilities for future application of the model.

REFERENCES

1. Frank, O. Die Grundform des arteriellen Pulses. Z. Biol. 1899, **37**, 483.
2. Aperia, A. Hemodynamical studies. Skand. Arch. Physiol. 1940, **83**, Suppl. 16.
3. Brotmacher, L. Evaluation of derivation of cardiac output from blood pressure measurements. Circulat. Res. 1957, **5**, 589.
4. Bridwell, T., Jenss, R. M., and Greene, D. G. An evaluation of Starr's equation for the prediction of stroke volume. Circulation 1956, **14**, 250.
5. Roston, S. Mathematical formulation of cardiovascular dynamics by use of the Laplace transform. Bull. math. Biophys. 1959, **21**, 1.
6. Leight, L., Snider, T. H., Clifford, G. O., and Hellems, H. K. Hemodynamic studies in sickle cell anemia. Circulation 1954, **10**, 653.
7. Salans, A. H., Katz, L. N., Graham, G. R., Gordon, A., Elisberg, E. I., and Gerber, A. A study of the central and peripheral arterial pressure pulse in man; correlation with simultaneously recorded electrokymograms. Circulation 1951, **4**, 510.
8. Remington, J. W., Noback, C. R., Hamilton, W. F., and Gold, J. J. Volume elasticity characteristics of the human aorta and prediction of the stroke volume from the pressure pulse. Amer. J. Physiol. 1948, **153**, 298.
9. Landowne, M., Brandfonbrener, M., and Shock, N. W. The relation of age to certain measures of performance of the heart and the circulation. Circulation 1955, **12**, 567.
10. Hamilton, W. F., and Dow, P. Experimental study of the standing waves in the pulse propagated through the aorta. Amer. J. Physiol. 1939, **125**, 48.

THE RENAL CLEARANCES OF IODIDE AND L-DIIODOTYROSINE MEASURED SIMULTANEOUSLY *

By WILLIAM E. LASSITER † AND JOHN B. STANBURY

(From the Department of Medicine, Harvard Medical School and the Medical Services of the Massachusetts General Hospital, Boston, Mass.)

(Submitted for publication October 13, 1958; accepted January 5, 1959)

After the intravenous administration of I^{131}-labeled L-diiodotyrosine (DIT), an increasing fraction of the labeled iodine of the serum becomes inorganic iodide as the DIT undergoes deiodination. Most of the labeled iodine which appears in the urine is inorganic iodide (1). If one makes an estimate of the renal clearance of inorganic iodide, based on the concentrations of labeled inorganic iodide in serum and urine after the administration of DIT, then values considerably in excess of those reported for iodide (2–5) are obtained. This could occur if DIT undergoes further deiodination after it enters the kidney.

The studies reported here are concerned with simultaneous measurements of the renal clearance of iodide and DIT, using a double-labeling technique with I^{133}-labeled iodide and I^{131}-labeled DIT. It is shown that the apparent renal clearance of I^{131}-labeled DIT is considerably less than the true clearance and, thus, that intrarenal deiodination of DIT does indeed occur.

MATERIALS AND METHODS

DIT labeled with I^{131} was prepared by iodination of L-tyrosine by the method of Roche, Lissitzky, Michel and Michel (6). I^{133} in the form of iodide was obtained from Brookhaven National Laboratories. The major component of the gamma radiation of I^{133} has an energy of 0.53 MEV. This is sufficiently different from the major I^{131} gamma energy of 0.36 MEV to permit differentiation of the two isotopes in a well-type scintillation counter with pulse-height analyzer.

Five subjects were selected for study. All were healthy males with no clinical evidence of renal disease or prostatism. The ages ranged from 29 to 63 years. In three of the subjects there was no thyroidal uptake of iodide because of previous thyroidectomy or medications, and in the other two (Subjects 2 and 5), uptake of iodine by the gland was blocked with a single 30 mg. dose of methimazole. All subjects were clinically euthyroid at the time of the study.

Priming doses of 50 μc. each of I^{131}-labeled DIT and of I^{133} as iodide were given intravenously, and a maintenance solution containing 40 μc. of DIT and 20 μc. of iodide^{133} in 50 ml. saline was administered by continuous I.V. drip at the rate of 2 ml. per minute. After a 30 minute period for equilibration, urine collections were made for four consecutive 30 minute periods, with a blood sample at the midpoint of each period. The labeled iodine in aliquots of each urine and serum sample was determined in the well-type scintillation counter at discriminator settings for I^{131} and I^{133}. Urine was then applied with carrier iodide and DIT directly to Whatman No. 3 filter paper for descending chromatography in butanol-20 per cent acetic acid. Because of the low concentration of labeled iodine in the serum samples, 2 ml. aliquots were first acidified to pH 2 with H_2SO_4 and thrice extracted with butanol saturated with 5 per cent $Na_2S_2O_3$. The butanol extracts were evaporated to dryness at 65° C. and redissolved in ethanol NH_3 prior to chromatography. After chromatography the strips were sprayed with palladium chloride and diazotized sulfanilic acid solutions to identify the iodide and DIT markers. Strips were then cut in segments and counted both for I^{131} and I^{133} in the well counter. Inulin clearances were simultaneously determined by the conventional method in three of the five patients.

In order to test the accuracy of the chromatographic method for measuring the relative quantities of DIT and iodide, normal serum was incubated with four different known ratios of DIT and iodide. Samples of each were extracted, chromatographed and measured as described. The results, shown in Table I, indicate that the method

TABLE I

Recovery of known amounts of I^{131}-labeled DIT and iodide after incubation with serum *

Sample	Labeled iodine added as inorganic iodide	Labeled iodine recovered	Labeled iodine recovered as inorganic iodide
no.	%	%	%
1	24.1	73.5	22.6
2	41.7	68.3	42.2
3	61.0	66.0	60.2
4	80.1	70.2	79.0

* Three successive 10 ml. butanol extractions of 2 ml. samples were chromatographed.

* Supported in part by Grant No. A-1880 of the Division of Arthritis and Metabolic Diseases of the United States Public Health Service.

† Recipient of a Donner Fellowship for Medical Research. Present address: Department of Medicine, University of North Carolina, Chapel Hill, N. C.

provides a satisfactory indication of the ratio of concentration of the labeled substances in the serum samples. Losses in the processes of extraction, transfer and chromatography were random and did not affect the ratio after three extractions.

Computations. Because the I^{133} available is contaminated with I^{131}, it is necessary to apply a correction to the measured values of the labeled iodine in urine and serum at the two settings. This can be done by measuring known standards of I^{131} and I^{133} at the two settings on the pulse-height analyzer. Simultaneous equations can then be set up and solved as follows:

$$A = x + ay$$
$$B = bx + y$$

where

A = observed counts of sample at I^{131} setting,
B = observed counts of sample at I^{133} setting,
x = true I^{131} counts,
y = true I^{133} counts,

$$a = \frac{\text{counts of } I^{133} \text{ standard at } I^{131} \text{ setting}}{\text{counts of } I^{133} \text{ standard at } I^{133} \text{ setting}},$$

and

$$b = \frac{\text{counts of } I^{131} \text{ standard at } I^{133} \text{ setting}}{\text{counts of } I^{131} \text{ standard at } I^{131} \text{ setting}}.$$

The values of x and y determined by the solution of these equations can then be applied directly to the computation of the renal clearances (C) of I^{131} and I^{133}, *viz.*

$$C_{I^{133}} = \frac{\text{corrected urine } I^{133} \text{ counts} \times \text{volume of urine}}{\text{corrected serum } I^{133} \text{ counts} \times \text{duration of collection}}$$

provided equal volumes of urine and serum are counted. The $C_{I^{133}}$ was a true measure of the renal clearance of iodide, since it was shown by chromatography that all of the I^{133} in both serum and urine was present solely as iodide. The overall clearance of I^{131}, which can be computed in similar fashion, is a function of both the iodide and DIT clearances.

The fraction of the total I^{131} present as iodide and as DIT was determined by chromatography of urine and serum samples. From these data, an apparent renal clearance of DIT can be computed which is related to the overall clearance of I^{131} ($C_{I^{131}}$) as follows:

$$\text{App. } C_{DIT} = \frac{f_{DIT} \text{ urine}}{f_{DIT} \text{ serum}} \times C_{I^{131}}$$

where f_{DIT} urine equals the fraction of total urinary I^{131} present as DIT and f_{DIT} serum equals the fraction of total serum I^{131} present as DIT. f_{DIT} urine is always small. Similarly, an apparent renal clearance of iodide can be computed.

The true clearance of DIT is the apparent clearance plus an amount of DIT equivalent to any iodide which may have been split from DIT during passage through the kidney. Thus, the last term of Equation *1* below is an expression to add to the clearance value that iodide in the urine which was derived by breakdown of DIT in the kidney. If there were no breakdown in the kidney, the numerator of this term would vanish.

1) $$\text{True } C_{DIT} = \frac{U_{DIT}}{S_{I^{131}} \cdot f_{DIT \text{ serum}} \cdot T} + \frac{U_{I^{131}-} - S_{I^{131}-} \cdot C_{I^{133}} \cdot T}{S_{I^{131}} \cdot f_{DIT \text{ serum}} \cdot T}$$

2) $$\text{True } C_{DIT} = \frac{U_{DIT} + U_{I^{131}-}}{S_{I^{131}} \cdot f_{DIT \text{ serum}} \cdot T} - \frac{S_{I^{131}-} \cdot C_{I^{133}} \cdot T}{S_{I^{131}} \cdot f_{DIT \text{ serum}} \cdot T}$$

3) $$C_{I^{131}} = \frac{U_{DIT} + U_{I^{131}-}}{S_{I^{131}} \cdot T}$$

4) $$\text{True } C_{DIT} = \frac{C_{I^{131}} - f_{I^{131}-} \cdot C_{I^{133}}}{f_{DIT \text{ serum}}}$$

where

U_{DIT} = total labeled DIT in urine during a collection period,
$U_{I^{131}-}$ = urine labeled inorganic iodide,
S_{DIT} = serum labeled DIT per unit volume,
$S_{I^{131}-}$ = serum labeled inorganic iodide,
$S_{I^{131}}$ = total serum I^{131},
$C_{I^{133}}$ = total clearance of I^{133}, or inorganic labeled iodide clearance,
$f_{DIT \text{ serum}}$ = fraction of serum I^{131} present as DIT,
$f_{I^{131}-}$ = fraction of serum I^{131} present as inorganic iodide, and by definition equal to $S_{I^{131}-}/S_{I^{131}}$,

and

T = time.

RESULTS AND DISCUSSION

Values for $C_{I^{133}}$, $C_{I^{131}}$ and the apparent and true clearances of DIT for each of the five subjects are summarized in Table II. In Subject No. 5, the first collection period was discarded because an equilibrium state had not been reached, and C_{DIT} could not be computed for the final period because of laboratory contamination of the chromatograms. Considerable individual variation in the renal clearance of iodide ($C_{I^{133}}$) was noted. The highest values were found in younger subjects. In the two youngest subjects, clearance values somewhat higher than those recorded in the literature (*2–5*) were found. There was also considerable variation in the apparent and true clearance of DIT, the ratios varying from 0.2 to 0.55. This is presumably attributable to varying degrees of deiodination of DIT in the kidney.

In each case, the true clearance of DIT was found to be greater than the apparent clearance, as estimated from serum and urine DIT concentrations. Thus, postglomerular, presumably intrarenal, deiodination of DIT must have occurred. This could have taken place either by deiodination of filtered DIT, or by direct secretion of de-

TABLE II

Renal clearance of diiodotyrosine (DIT) and of iodide in five male subjects *

Period	$C_{I^{131}}$	Error†	$C_{I^{133}}$ (iodide)	Error†	Apparent C_{DIT}	Error†	True C_{DIT}	Error‡	Inulin clearance
	ml./min.		*ml./min.*		*ml./min.*		*ml./min.*		*ml./min.*
Pt. No. 1 40 M									
1	23.2	±0.6	38.2	±2.2	3.2	±0.4	20.2	±2.3	
2 (60 min.)	20.4	±0.5	28.5	±1.6	3.3	±0.4	15.3	±1.7	
3	21.5	±0.6	27.8	±1.8	3.6	±0.5	16.9	±1.9	
Average	21.4		30.8		3.4		16.9		
Pt. No. 2 36 M									
1	28.8	±0.9	51.0	±2.7	4.2	±0.6	17.7	±2.9	
2	38.6	±1.2	65.9	±3.3	9.5	±1.8	22.4	±3.5	
3	38.2	±1.0	56.7	±2.6	6.6	±1.2	25.7	±2.8	
4	27.3	±0.7	40.4	±1.8	5.0	±0.9	13.5	±1.9	
Average	33.2		53.5		6.3		19.8		
Pt. No. 3 63 M									
1	17.5	±0.4	29.8	±1.5	5.0	±0.6	11.8	±1.6	77.0
2	17.6	±0.4	29.0	±1.7	4.1	±0.8	11.1	±1.8	83.8
3	15.1	±0.4	23.1	±1.5	2.9	±0.6	10.0	±1.6	68.8
4	17.2	±0.4	24.4	±1.6	3.7	±0.8	11.1	±1.7	84.0
Average	16.8		26.6		3.9		11.0		78.1
Pt. No. 4 29 M									
1	40.5	±0.8	65.5	±3.1	8.8	±0.9	28.8	±3.2	103.4
2	42.8	±0.9	69.6	±3.6	7.2	±0.9	23.2	±3.7	90.7
3	46.4	±1.1	68.0	±3.6	6.1	±0.9	30.2	±3.8	84.9
4	50.3	±1.4	71.1	±4.6	9.4	±2.4	31.3	±4.8	77.5
Average	45.0		68.5		7.8		28.4		89.1
Pt. No. 5 60 M									
1	19.9	±0.7	30.1	±1.7	5.2	±1.4	7.4	±1.8	69.6
2	21.0	±0.7	29.0	±1.6	4.8	±1.4	10.8	±1.7	59.2
3	17.6	±0.7	25.9	±1.4					53.2
Average periods 2–3	20.4		29.5		5.0		9.1		64.4

* $C_{I^{131}}$ is the observed overall clearance of I^{131} after administration of I^{131}-labeled DIT. $C_{I^{133}}$ is the clearance of I^{133} administered simultaneously as inorganic iodide. C_{DIT} is the clearance of DIT.

† Errors of counting only expressed as standard deviation.

‡ Square root of sum of square of I^{131} and I^{133} counting errors.

rived iodide by the cells of the kidney tubules. Deiodination in the ureters or bladder could conceivably occur but seems less likely. Deiodination did not occur in urine samples which were allowed to stand overnight at room temperature.

For any clearance studies to be valid, it is necessary that the substance being studied remain intact. DIT undergoes deiodination in the process of excretion; hence, it is not possible to estimate its true clearance by a single tracer technique. The true clearance can be calculated only if an independent measurement of the iodide clearance is obtained simultaneously, as by the double tracer technique described here.

SUMMARY AND CONCLUSIONS

1. Simultaneous determinations of the renal clearance of I^{131}-labeled diiodotyrosine and I^{133}-labeled iodide have been performed. Differential measurement of the two isotopes was found to be technically feasible.

2. The apparent renal clearance of diiodotyrosine is lower than the true clearance because of intrarenal deiodination of diiodotyrosine.

ACKNOWLEDGMENTS

The authors wish to acknowledge their indebtedness to Dr. Gordon Brownell and Dr. Alexander Leaf for help and advice, and for the inulin determinations. Dr.

Pablo Fletcher assisted in the observations on the first two patients.

REFERENCES

1. Stanbury, J. B., and Litvak, J. The metabolism of iodotyrosines. IV. Metabolism of diiodotyrosine in patients with hypothyroidism. J. clin. Endocr. 1957, **17**, 654.
2. Myant, N. B., Pochin, E. E., and Goldie, E. A. G. The plasma iodide clearance rate of the human thyroid. Clin. Sci. 1949, **8**, 109.
3. McConahey, W. M., Keating, F. R., Jr., and Power, M. H. An estimation of the renal and extrarenal clearance of radioiodide in man. J. clin. Invest. 1951, **30**, 778.
4. Perry, W. F., and Hughes, J. F. S. The urinary excretion and thyroid uptake of iodine in renal disease. J. clin. Invest. 1952, **31**, 457.
5. Becker, D. V., and Danzig, L. E. Kinetics of radioiodine distribution in chronic renal disease studied by means of the artificial kidney. Trans. Amer. Goiter Ass. 1954, 301.
6. Roche, J., Lissitzky, S., Michel, O., and Michel, R. Étude radiochromatographique des étapes de iodu-ration de la tyrosine et de l'histidine. Biochim. biophys. Acta 1951, 7, 439.

THE EFFECT OF TRIIODOTHYRONINE ON THE OXIDATIVE METABOLISM OF ERYTHROCYTES. I. CELLULAR STUDIES * †

By THOMAS NECHELES AND ERNEST BEUTLER

(From the Argonne Cancer Research Hospital ‡ and the Department of Medicine, The University of Chicago, Chicago, Ill.)

(Submitted for publication September 8, 1958; accepted January 9, 1959)

The mature, non-nucleated mammalian erythrocyte is a useful system in which to study the control of both anaerobic glycolysis and the hexose monophosphate shunt in an intact-cell preparation. The physiologic and metabolic properties of this cell have been reported extensively (1, 2). The final suspension can be obtained conveniently in relatively pure culture from man or experimental animals, and the quantity of cells studied can be measured easily. Inasmuch as the erythrocyte has only slight remnants of the citric acid cycle, cytochrome system, and pathway for protein synthesis, these cannot be evaluated. However, the cell can carry out the reactions of the glycolytic pathway and of the hexose monophosphate shunt. When the erythrocyte is incubated with methylene blue, the hexose monophosphate shunt is activated by increasing the rate of oxidation of reduced triphosphopyridine nucleotide (TPNH). The rate of metabolism via this path can be estimated by determining the rate of oxygen consumption of the cells (3, 4).

This report describes the use of this system in an attempt to define the locus of action of the thyroid hormone.

MATERIALS AND METHODS

Reagents. Glucose-6-phosphate (G-6-P), triphosphopyridine nucleotide (TPN), 3,5,3′-triiodo-L-thyronine (TRITh), L-thyroxine, 3,5-diiodo-L-thyronine (DITh), 3,5-diiodo-L-tyrosine (DIT), and 3,5-dinitro-L-tyrosine (DNT) were obtained from Sigma Chemical Co. The disodium salt of ethylenediaminetetraacetic acid (Disodium Versene®) was obtained from Versenes, Inc. All of these reagents were dissolved in 0.9 per cent sodium chloride solution prior to use. A few drops of 1 N NaOH were used and the solution was heated to 55° C. to aid solution of TRITh when used in the highest concentration, 3.75×10^{-6} M. The buffered saline used in these experiments was prepared by the method outlined by Umbreit, Burris and Stauffer (5). The buffer was prepared by dissolving 1.78 Gm. of $Na_2HPO_4 \cdot 2H_2O$ in about 90 ml. of distilled water. The pH was adjusted to 7.4 with approximately 2.0 ml. of 1 N HCl, and sufficient water was added to bring the total volume to 100 ml. Twenty ml. of this buffer was added to 109 ml. of 0.9 per cent NaCl. The final pH in all reaction flasks before incubation was 7.4; the fall during incubation was not more than 0.2 pH units.

Glucose-1-C^{14} and glucose-6-C^{14} were obtained from Volk Radiochemical Co. The labeled components were added to the reaction vessels together with carrier glucose to a final concentration of 5.55×10^{-4} M. The $C^{14}O_2$ evolved on incubation was absorbed in 10 per cent sodium hydroxide. The carbonate was precipitated and plated as barium carbonate, following the method described by Krahl (6). All counts were corrected for self-absorption and coincidence (7) and are expressed as total cpm for the $C^{14}O_2$ formed per flask in 90 minutes.

Glucose was determined as total reducing substance by the method of Folin and Malmros as described in Umbreit (5). Aliquots were removed at intervals and the results were calculated as μg. glucose consumed per ml. erythrocytes. Appropriate corrections were made (5) when glucose-6-phosphate was used as substrate.

Blood from normal human volunteers was drawn into a flask containing 0.5 ml. (500 units) heparin solution and was immediately centrifuged at $3,000 \times G$ for 10 minutes. The plasma and buffy coat were discarded, and the red cells were resuspended in an equal volume of cold buffered saline. The cells were put into the Warburg flasks within 30 minutes after they had been drawn. For studies in which hemolysates were used, the packed red cells were resuspended in an equal volume of buffered saline containing 478 mg. per cent nicotinamide to reduce hydrolysis of the nucleotides (8). The suspension was then hemolyzed by freezing and thawing three times. Aliquots equal to 1 ml. packed cells were used in all experiments.

Oxygen consumption was determined in a Warburg respirometer. Each flask contained approximately 1 ml. of erythrocytes and buffered saline, pH 7.4, in a total volume of 3.6 ml. Unless otherwise noted, the final

* Based on a dissertation submitted for the Master's degree in the Department of Physiology, The University of Chicago.

† Presented before the 31st annual meeting of the Central Society for Clinical Research, October, 1958.

‡ Operated by The University of Chicago for the U. S. Atomic Energy Commission.

TABLE I

Oxygen and glucose consumption of human erythrocytes in the presence of triiodothyronine (TRITh)

Experiment	Flask pairs	Oxygen consumption		p*	Glucose consumption	
		Control	TRITh		Control	TRITh
		µl./min./ml. erythrocytes			*µg./min./ml. erythrocytes*	
		Whole erythrocytes without methylene blue				
1	3	0.18	0.19			
2	3	0.43	0.46			
9	2	0.24	0.32			
16	2	0.44	0.61			
21	3	0.20	0.27	0.002		
		Whole erythrocytes plus methylene blue				
1	3	1.65	1.79			
2	2	1.83	2.05			
3	3	3.07	3.56			
7	3	2.18	2.71			
8	3	2.64	2.82			
9	4	1.80	2.02			
10	3	2.45	2.61			
11	3	2.57	3.04			
12	3	2.63	2.81			
13	3	2.17	2.49			
14	3	3.14	3.45			
15	3	1.93	2.85		17.8	24.1
16	4	2.00	2.21		18.5	23.4
18	3	3.04	3.55		22.1	26.3
21	3	1.25	1.41		12.2	12.2
22	3	4.10	4.83	0.001	30.4	32.9
		Hemolysates plus methylene blue				
4	3	1.38	2.40			
5	3	2.55	3.43			
6	3	1.37	2.49			
7	3	2.04	2.84			
13	3	2.98	3.21			
23	3	1.82	2.48		15.7	21.4
24	3	1.47	1.82			
25	3	1.93	2.58	0.002		

* Calculated by student's "t" test on the basis of per cent increase in paired individual flasks within each experiment.

concentrations were: 3.75×10^{-3} M TRITh, 10^{-4} M methylene blue (MeB), and 5.55×10^{-4} M glucose. The flasks and contents were equilibrated under air for 10 minutes at 37° C. in the water bath, and the oxygen consumption was then measured at 10 to 15 minute intervals for 90 minutes. When hemolysates were used, 5.55×10^{-4} M glucose-6-phosphate was substituted for the glucose. Microhematocrit or hemoglobin determinations were carried out on all flasks. Oxygen uptake was expressed as µl. per minute per ml. cells.

RESULTS

Table I summarizes the data obtained when erythrocytes were incubated with TRITh. As has been reported repeatedly (9), the oxygen consumption of mature mammalian erythrocytes in the absence of methylene blue was extremely small. Upon incubation with TRITh, the rate of oxygen utilization increased slightly.

In the presence of methylene blue, human erythrocytes incubated with physiologic amounts of TRITh exhibited consistently a small but statistically highly significant increase in the rate of oxygen consumption (Table I). Hemolysates of cells from the same donors incubated with glucose-6-phosphate revealed similar or greater stimulation when TRITh was added. The data obtained from a representative experiment are presented in Figures 1 and 2. When the concentration of TRITh was increased 100-fold, to 3.75×10^{-6} M, more than 100 per cent stimulation of O_2 consumption was demonstrated. The relationship between the magnitude of stimulation observed and the concentration of TRITh in the reaction mixture is illustrated in Figure 3.

In five out of six experiments, glucose consump-

tion was found to increase comparably to the increases in oxygen consumption (Table I).

Previous workers have demonstrated that the oxidation of glucose, which occurs in erythrocytes when incubated with methylene blue, involves carbons 1–3 and occurs in the hexose monophosphate shunt (4). In order to determine whether increased oxygen consumption represents stimulation of metabolism in this pathway, rather than alternate pathways of oxidative metabolism, specifically labeled glucose was added to the red cell suspension. Incubation was carried out with and without methylene blue and with and without TRITh. The radioactivity of the carbonate obtained from cells incubated with glucose-1-C^{14} and glucose-6-C^{14} was compared. The results (Table II) indicated that carbon-1 was preferentially oxidized, supporting the interpretation that the hexose monophosphate shunt was stimulated by TRITh.

To determine whether the stimulation of metabolism in this pathway paralleled the known metabolic activities of these compounds, further studies were carried out with substances related to triiodothyronine: thyroxine diiodotyrosine, dinitrotyrosine, and inorganic iodide were tested in comparable concentrations. The results are shown in Tables II and III, and in Figure 4. In concentrations up to 20 times that of TRITh used in these experiments (7.5×10^{-7} M *vs.* 3.75×10^{-8} M), none of the compounds except L-thyroxine produced significant stimulation. Although stimulation was demonstrated when thyroxine was added, it was much less than that observed with comparable concentrations of TRITh. This finding agrees with the comparative *in vivo* activities of these compounds. Evaluation of stimulation using iodinated thyronine analogues of TRITh (*i.e.*, diiodothyronine and monoiodothyronine) could not be made because the commercially obtainable preparations of these compounds are not altogether free of the tri- and tetra-iodo-compounds and thus show biologic activity. The chelating agents, citrate and Versene®, were devoid of stimulating activity (Figure 4).

It has been suggested that the thyroxine analogues may act as accessory electron transporting

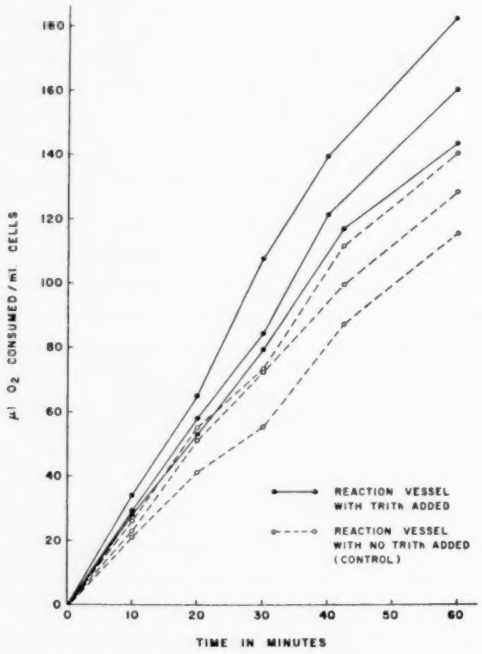


FIG. 1. THE EFFECT OF TRIIODOTHYRONINE (TRITH) ON THE OXYGEN CONSUMPTION OF ERYTHROCYTES

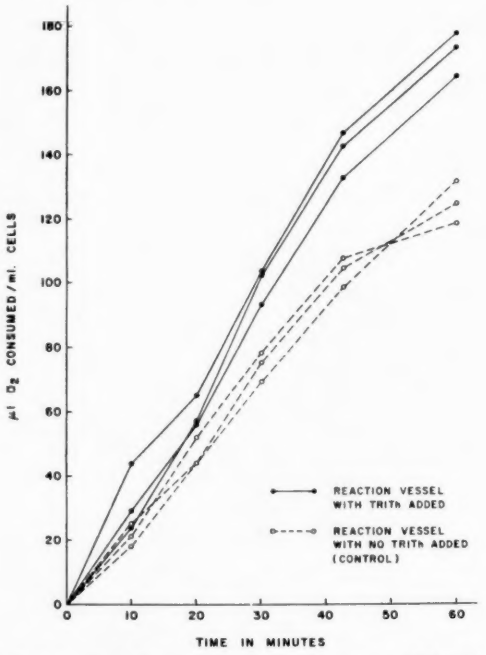


FIG. 2. THE EFFECT OF TRIIODOTHYRONINE (TRITH) ON THE OXYGEN CONSUMPTION OF HEMOLYSATES PREPARED FROM THE SAME BLOOD SAMPLE

TABLE II

The production of $C^{14}O_2$ by erythrocytes incubated with methylene blue

Experiment	Sample*	Oxygen consumption	Cpm from: Glucose-1-C^{14}	Glucose-6-C^{14}	Cpm added to flask
		µl./min./ml. erythrocytes			
9	Cells	0.24	3,573	135	1.9×10^5
	Cells, TRITh	0.32	3,240	117	
	Cells, MeB	1.80	95,044	285	
	Cells, MeB, TRITh	2.02	120,385	145	
14	Cells, MeB	3.14	1,129		1.3×10^4
	Cells, MeB, TRITh	3.44	1,315		
	Cells, MeB, DIT	3.15	1,125		
	Cells, MeB, DNT	3.06	1,120		
	Cells, MeB, KI	3.25	1,140		
15	Cells, MeB	1.93	1,880		4.7×10^4
	Cells, MeB, TRITh	2.58	2,493		
	Cells, MeB, DIT	2.25	1,908		
	Cells, MeB, DNT	2.02	1,661		
	Cells, MeB, KI	2.19	1,860		
16	Cells, MeB	2.00	28,908	135	4.7×10^5
	Cells, MeB, TRITh	2.21	32,320	199	
18	Cells, MeB	3.04	50,130		4.7×10^5
	Cells, MeB, TRITh	3.55	54,591		
	Cells, MeB, DIT	2.91	50,100		
	Cells, MeB, KI	3.28	50,073		

* Abbreviations are as follows: TRITh, 3,5,3′-triiodo-L-thyronine; MeB, methylene blue; DIT, 3,5-diiodo-L-tyrosine; DNT, 3,5-dinitro-L-tyrosine; KI, potassium iodide.

p value calculated by student's "t" test on the basis of per cent increase in counts per minute in paired individual flasks within each experiment.

	TRITh	DIT	KI
p =	0.003	>0.50	>0.50

mechanisms, perhaps replacing TPN (10, 11). To evaluate the importance of an intact electron transferring system in the mechanism of TRITh stimulation, the following experiment was carried

TABLE III

The effect of various analogues of TRITh upon oxygen consumption of erythrocytes incubated in methylene blue *

Experiment	No. of flask pairs	Oxygen consumption: Control	TRITh	DIT	KI
			µl./min./ml. cells		
10†	3	2.45	2.61	2.43	2.50
11	3	2.57	3.04	2.70	2.63
14	3	3.14	3.45	3.15	3.25
15	3	1.93	2.58	2.25	2.19
18	3	3.04	3.55	2.91	3.28
p			<0.001	>0.30	0.10

* Abbreviations and concentrations are as follows: TRITh, 3,5,3′-triiodo-L-thyronine, 3.75×10^{-8} M; DIT, 3,5-diiodo-L-tyrosine, 7.5×10^{-7} M; DNT, 3,5-dinitro-L-tyrosine, 7.5×10^{-7} M; KI, potassium iodide, 7.5×10^{-7} M.

† TRITh and other compounds were all tested at the same concentration, 3.75×10^{-8} M, in this experiment.

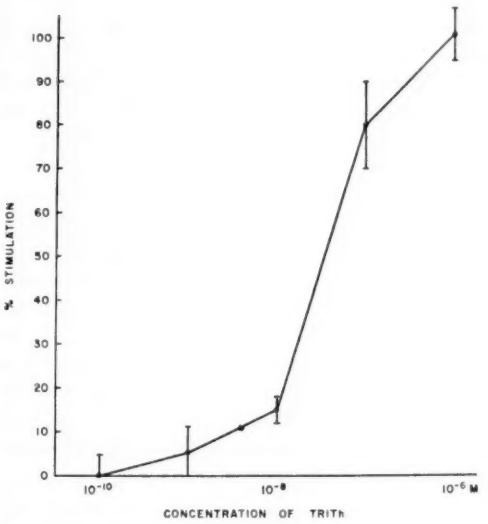


FIG. 3. STIMULATION OF OXYGEN CONSUMPTION VS. CONCENTRATION OF TRIIODOTHYRONINE (TRITH) IN REACTION MIXTURE

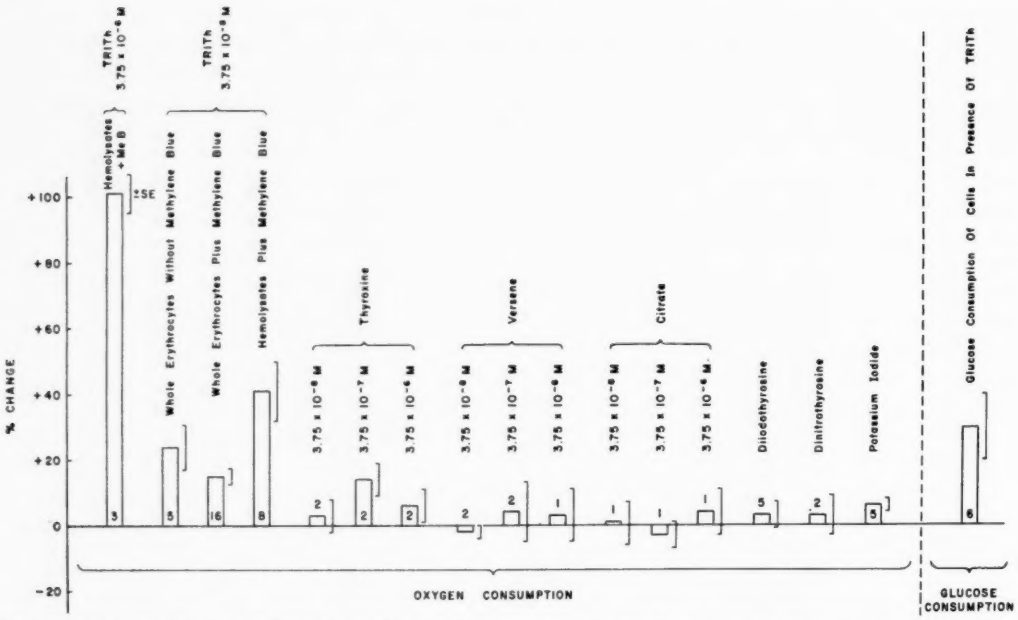


FIG. 4. PER CENT CHANGE IN OXYGEN AND GLUCOSE CONSUMPTION IN THE PRESENCE OF TRIIODOTHYRONINE (TRITH) AND ANALOGUES

Average of number of experiments indicated.

out. Erythrocytes were hemolyzed in the absence of nicotinamide. This promotes hydrolysis of TPN (8). Aliquots of the hemolysate were incubated with glucose-6-phosphate and methylene blue in Warburg reaction vessels. TRITh was added to one-half of the samples, and oxygen consumption was measured for 60 minutes. At the end of this period, TPN was tipped into the reaction mixture to give a final concentration of 2.5×10^{-7} M, and oxygen consumption was measured for an additional 30 minutes. Before the addition of TPN, no difference in oxygen consumption was observed (0.04 *vs.* 0.04 μl. oxygen per minute per ml. cells) in the presence or absence of TRITh. However, when TPN was introduced into the system, the rate of oxygen utilization returned to normal and stimulation of O_2 utilization was observed in the presence of TRITh (4.81 *vs.* 3.53 μl. oxygen per minute per ml. cells). The results are shown in Figure 5.

DISCUSSION

The mechanisms involved in the actions of the hormones upon specific target organs has long been a subject of speculation. Within the past several years, experimental evidence has been obtained regarding the possible mechanisms of action of several hormones. Among these are epinephrine (12), adrenocorticotropic hormone (ACTH) (13), and estradiol (14). The site of action of the thyroid hormone has not been elucidated, however. The studies reported here represent the first part of an investigation of the locus of action of this hormone.

Because of the relative simplicity of the mammalian erythrocyte, this cell was chosen in an attempt to localize some of the immediate metabolic effects of TRITh. This form of thyroid hormone was chosen because it acts more rapidly than thyroxine *in vivo*. The effects of thyroid hormone or its analogues has not been studied previously *in vitro* in the red blood cell. Erythrocytes have been found to have an increased rate of glycolysis when thyroxine was administered to experimental animals, but no attempt was made to investigate the mechanism of this action (15). In the present investigation, it has been shown that

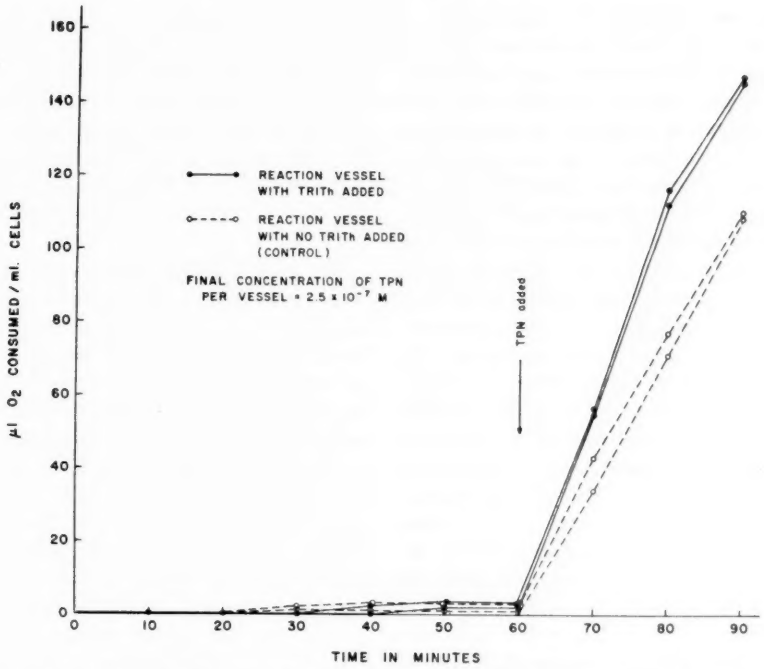


FIG. 5. STIMULATION OF OXYGEN CONSUMPTION OF HEMOLYZED ERYTHROCYTES BY TRIIODOTHYRONINE TRITH IN THE PRESENCE AND ABSENCE OF TRIPHOSPHOPYRIDINE NUCLEOTIDE (TPN)

TRITh stimulates the oxygen consumption of human erythrocytes *in vitro*.

Because so many of the mechanisms proposed to explain the action of thyroxine on the basis of *in vitro* experimental evidence have been shown to be the result of rather nonspecific action on various systems (16), several metabolically inert structural analogues of TRITh were tested. DIT and DNT were found to have little or no effect, both in terms of oxygen consumption and $C^{14}O_2$ production, even though they were present in concentrations 20 times that of TRITh. The same was found to be true for inorganic iodide and the chelating agents, Versene® and citrate. In addition, it was found that although thyroxine produced stimulation of oxygen consumption, concentrations required were much higher than those of TRITh, an observation which agrees well with the comparative *in vivo* action of these compounds.

Several alternative mechanisms have been suggested to explain how thyroxine increases oxygen consumption. These proposed mechanisms can be classified into two broad categories: *1*) those that postulate a direct or primary action on the permeability characteristics of one or more of the cellular and intracellular barriers, and *2*) those that postulate an action on one or more enzyme systems.

Several workers (17) have proposed that the specific action of thyroxine is to change the permeability characteristics of the mitochondria, producing swelling and thus changing the surface organization of these subcellular particles. However, it is immediately apparent that such a mechanism is not the operative one in the system that we have studied. Mitochondria have not been demonstrated to exist in mature erythrocytes, and TRITh produces the stimulation even after the removal of the physiologically intact membrane (by hemolysis). It seems more probable that, as suggested by the work of Lehninger and Ray (18), the swelling observed in mitochondria exposed to thyroxine *in vivo* or *in vitro* is directly related to the oxidation-reduction state of the mitochondrial electron carrier system. Thus, while the thyroid

hormone may well influence the permeability of membranes, this is apparently an indirect effect.

Direct action of the hormone upon individual enzymes or enzyme systems has often been suggested as an alternative possibility for the mode of action of the thyroid hormone at the subcellular level. Various enzymes have been found to be affected by the *in vivo* administration of thyroxine. Among these are intestinal glucokinase (19), acyl phosphatase (20), succinoxidase, glutamic dehydrogenase, cytochrome oxidase (21), DPNH-oxidase (22), steroid reductase (23, 24), glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase (25), TPNH-cytochrome C reductase (26), and hexokinase (17). Data obtained by such means are always difficult to interpret, since many indirect mechanisms may affect enzyme activity when thyroxine is administered to the animal. Although succinoxidase activity was found to be stimulated in mitochondrial preparations obtained from thyroxine-injected animals (27), no direct action of the hormone was found in submitochondrial preparations. Wolff, Wolff and Ball, on the other hand (28, 29), report inhibition of various dehydrogenases by thyroxine and various analogues *in vitro*. Although they point out that this mechanism could increase the oxygen consumption of tissues *in vivo* by diverting metabolites into primarily oxidative pathways, they question the actual operation of this mechanism under physiologic conditions.

In the intact organism and in most tissues, several routes for oxygen utilization are available. In the human erythrocyte, on the other hand, oxygen utilization occurs almost entirely through one pathway, the hexose monophosphate shunt. Metabolism through this pathway is enhanced in the presence of methylene blue. Since this is the only known pathway of oxygen consumption, we may presume that stimulation of oxygen consumption by TRITh is due to stimulation of metabolism in this pathway. The glucose consumption in the stimulated cells indicates that the increased metabolism via the hexose monophosphate pathway is not merely a secondary manifestation of inhibition of the glycolytic pathway. The finding that the increased CO_2 produced originated from the 1-carbon but not from the 6-carbon strongly supports the conclusion that stimulation of oxygen consumption occurred in the hexose monophosphate shunt. Other known mammalian oxidative pathways produce CO_2 from the 6- as well as the 1-carbon. Furthermore, the dissociation of phosphorylation and oxygen consumption observed in the intact animal is well explained by stimulation of the hexose monophosphate shunt since the oxidation of TPNH is not coupled to phosphorylation (30).

The steps through which substrate is thought to be oxidized in the hexose monophosphate pathway in the mature mammalian erythrocyte are illustrated in Figure 6. The studies reported here do not show what the exact mechanism of the stimulation of this pathway is. Our studies indicate that stimulation of oxygen consumption can be demonstrated only when TPN is present, but that it may be observed both in the presence and absence of methylene blue. These findings must be taken into account when further efforts are made to delineate clearly the site of action of the thyroid hormones in the human erythrocytes. It is hoped that further studies now in progress will provide an explanation of the action of thyroxine in this system on an enzymatic level.

It should be emphasized that the system used for these investigations is not typical of the majority of the cells affected by the thyroid hormone(s) and thus the mechanisms observed may not be general. More than one mechanism of action may exist in complex tissues. Using liver slices from thyrotoxic rats, Glock, McLean and Whitehead (31) have concluded, on the basis of rates of carbon dioxide production from specifically labeled glucose, that thyroxine enhances metabolism via the glycolytic pathway. However, samples were removed for counting only after a 90 minute interval. More recently Spiro and Ball (32), studying the rate of labeled carbon dioxide production in intact animals to which specifically labeled glucose had been administered, found an initial increase in the proportion of glucose carbon-1 oxidized to carbon dioxide. Within 30 minutes, however, the ratio of carbon-1 to carbon-6 in the expired carbon dioxide approached that found in the normal animal. From their data, these authors concluded that both the glycolytic and hexose monophosphate pathways were stimulated by thyroxine *in vivo*. As has been empha-

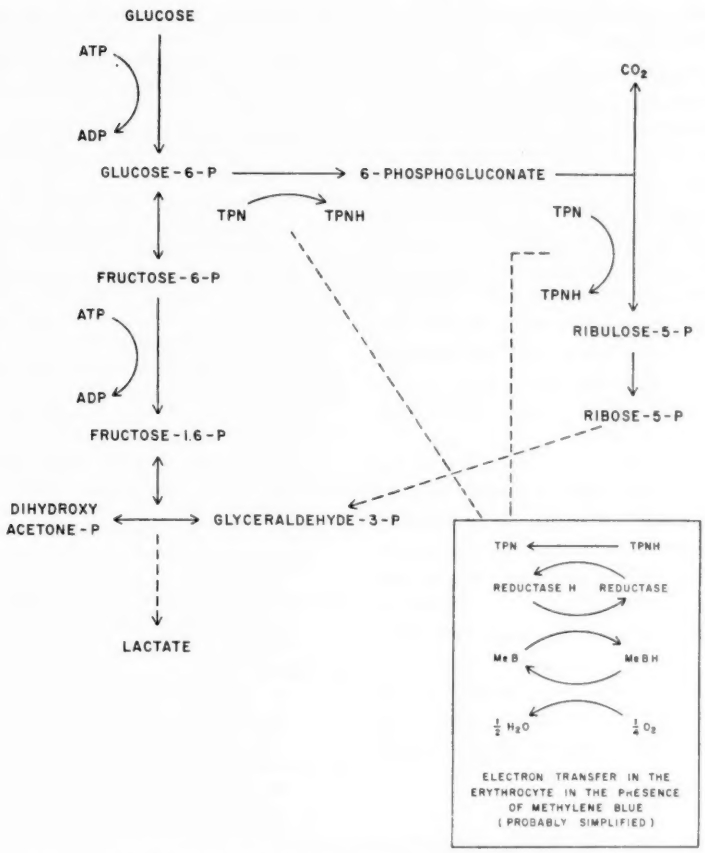


FIG. 6. SIMPLIFIED SCHEME OF CARBOHYDRATE METABOLISM IN THE HUMAN ERYTHROCYTE (1)

sized recently by Wood and Katz (33), however, after a short initial period randomization of the label occurs within the carbohydrate pools. In other words, once hexose has been decarboxylated to pentose, further metabolism of the pentose moiety takes place rapidly. Recycling is known to occur in the hexose monophosphate pathway and, in addition, further oxidation of triose fragments formed may occur by the Krebs' cycle reactions. Thus, over extended periods the appearance of carbon dioxide from specifically labeled glucose must be interpreted with caution. It is our opinion, therefore, that the data obtained by Spiro and Ball (32) would be entirely compatible with the interpretation that the primary locus of action of thyroid hormone in tissues other than the erythrocyte is in the hexose monophosphate pathway.

While it is certainly not permissible to extrapolate our findings in human erythrocytes to other cell types, these investigations may help to focus attention on the hexose monophosphate pathway as a possible locus of thyroid hormone action in other tissues.

SUMMARY

1. Significant stimulation of oxygen consumption of intact human erythrocytes incubated in the presence of glucose and methylene blue was observed upon the addition of physiologic amounts of triiodothyronine (TRITh), a more physiologically active analogue of thyroxine. The de-

gree of stimulation varied directly with the concentration of TRITh used.

2. Equal or greater stimulation of oxygen uptake was observed when hemolysates were incubated in the presence of methylene blue, glucose-6-phosphate, and TRITh, indicating that the observed effect was not due primarily to alterations in the transmembrane transfer of glucose or to changes in the rate of phosphorylation.

3. Glucose consumption was also increased when erythrocytes were incubated with TRITh.

4. This stimulation was reflected in an increased $C^{14}O_2$ production from glucose-1-C^{14} but not from glucose-6-C^{14}, suggesting that the increase in oxygen utilization was the result of stimulation of the hexose monophosphate shunt.

5. Stimulation observed with TRITh was not elicited by physiologically inactive analogues of the hormone including dinitrotyrosine, diiodotyrosine, and potassium iodide. Chelating agents, citrate and Versene®, also had no effect on oxygen consumption. Thyroxine showed some stimulatory effect when present in relatively high concentrations.

6. Triiodothyronine was not capable of replacing triphosphopyridine nucleotide (TPN) in the electron transferring system of the erythrocyte.

ACKNOWLEDGMENT

We wish to acknowledge the helpful advice of Dr. M. E. Krahl, Department of Physiology, The University of Chicago.

REFERENCES

1. Prankerd, T. A. J. The metabolism of the human erythrocyte (a review). Brit. J. Haemat. 1955, **16**, 131.
2. Granick, S. The chemistry and functioning of the mammalian erythrocyte. Blood 1949, **4**, 404.
3. Harrop, G. A., Jr., and Barron, E. S. G. Studies on blood cell metabolism; effect of methylene blue and other dyes on the oxygen consumption of mammalian and avian erythrocytes. J. exp. Med. 1928, **48**, 207.
4. Brin, M., and Yonemoto, R. H. Stimulation of the glucose oxidative pathway in human erythrocytes by methylene blue. J. biol. Chem. 1958, **230**, 307.
5. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques, 3rd ed. Minneapolis, Minn., Burgess Publishing Co., 1957.
6. Krahl, M. E. Oxidative pathways for glucose in eggs of the sea urchin. Biochim. biophys. Acta 1956, **20**, 27.
7. Aronoff, S. Techniques of Radiobiochemistry. Ames, Iowa, The Iowa State College Press, 1956.
8. Mann, P. J. G., and Quastel, J. H. Nicotinamide, cozymase and tissue metabolism. Biochem. J. 1941, **35**, 502.
9. Damble, K. Über die Atmung der roten Blutkörperchen bei Anämie. Z. ges. exp. Med. 1933, **86**, 595 and 608.
10. Gemmill, C. L. Discussion *in* The Thyroid, Brookhaven Symp. in Biol. Upton, N. Y., Brookhaven National Laboratory, 1955, No. 7, p. 97.
11. Gemmill, C. L. Enzymatic mechanisms of thyroxine. J. clin. Endocr. 1952, **12**, 1300.
12. Sutherland, E. W. The effect of hyperglycemic factor and epinephrine on enzyme systems of liver and muscle. Ann. N. Y. Acad. Sci. 1951, **54**, 693.
13. Haynes, R. C. Activation of adrenal phosphorylase by ACTH. Fed. Proc. 1958, **17**, 376.
14. Talalay, P., and Williams-Ashman, H. G. Activation of hydrogen transfer between pyridine nucleotides by steroid hormones. Proc. nat. Acad. Sci. (Wash.) 1958, **44**, 15.
15. Macho, L. The effect of thyroid hormone on the glycolytic activity of blood. Clin. chim. Acta 1957, **2**, 345.
16. Smith, R. H., and Williams-Ashman, H. G. The influence of thyroxine on the enzymatic activity of rat tissues. Biochim. biophys. Acta 1951, **7**, 295.
17. Tapley, D. F., and Cooper, C. Effect of thyroxine on the swelling of mitochondria isolated from various tissues of the rat. Nature (Lond.) 1956, **178**, 1119.
18. Lehninger, A. L., and Ray, B. L. Oxidation-reduction state of rat liver mitochondria and the action of thyroxine. Biochim. biophys. Acta 1957, **26**, 643.
19. Nishikawara, M. T. Thyroid control of glucokinase and glucose-6-phosphatase activity of rat intestinal mucosa. Fed. Proc. 1958, **17**, 118.
20. Harary, I. In vivo stimulation of acyl phosphatase by thyroxine. Fed. Proc. 1958, **17**, 237.
21. Tipton, S. R., Zirk, M., and St. Amand, G. S. Effect of thyroxine on mitochondrial enzyme activity in rat tissues. Fed. Proc. 1958, **17**, 174.
22. Brand, L., and Mahler, H. R. Effects of thyroxine and other factors on biogenesis of electron transport enzymes in chick embryos. Fed. Proc. 1958, **17**, 194.
23. Yates, F. E., Urkuhart, J., and Herbst, A. L. Effect of thyroid hormones on ring A reduction of cortisone by liver. Fed. Proc. 1958, **17**, 174.
24. McGuire, J. S. Effect of thyroxine on enzymatic steroid reduction. Fed. Proc. 1958, **17**, 272.
25. Glock, G. E., and McLean, P. A preliminary investigation of the hormonal control of the hexose monophosphate oxidative pathway. Biochem. J. 1955, **61**, 390.
26. Phillips, A. H., and Langdon, R. G. The activity of thyroxine and other hormones on hepatic TPN-

cytochrome reductase activity. Biochim. biophys. Acta 1956, **19**, 380.

27. Bronk, J. R. Action of thyroxine on oxidative phosphorylation in vitro. Fed. Proc. 1958, **17**, 196.
28. Wolff, J., and Wolff, E. C. The effect of thyroxine on isolated dehydrogenases. Biochem. biophys. Acta 1957, **26**, 387.
29. Wolff, E. C., and Ball, E. G. The action of thyroxine on the oxidation of succinate and malate. J. biol. Chem. 1957, **224**, 1083.
30. Kaplan, N. O., Swartz, M. N., Frech, M. E., and Ciotti, M. M. Phosphorylative and nonphosphorylative pathways of electron transfer in rat liver mitochondria. Proc. nat. Acad. Sci. (Wash.) 1956, **42**, 481.
31. Glock, G. E., McLean, P., and Whitehead, J. K. Pathways of glucose catabolism in rat liver in alloxan diabetes and hyperthyroidism. Biochem. J. 1956, **63**, 520.
32. Spiro, M. J., and Ball, E. G. A comparison of the pathways of glucose catabolism in the normal and hyperthyroid rat. J. biol. Chem. 1958, **231**, 31.
33. Wood, H. G., and Katz, J. The distribution of C^{14} in the hexose phosphates and the effect of recycling in the pentose cycle. J. biol. Chem. 1958, **233**, 1279.

PYRIMIDINE METABOLISM IN MAN. I. THE BIOSYNTHESIS OF OROTIC ACID *†

By LLOYD H. SMITH, JR. AND FAITH A. BAKER

(From the Huntington Memorial Laboratories and the Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Mass.)

(Submitted for publication November 12, 1958; accepted January 15, 1959)

Many studies of purine metabolism in man have been carried out. This is largely due to continued interest in the pathogenesis of gout and the ease of measuring serum and urinary uric acid. In contrast there have been few studies of pyrimidine metabolism in man. The major degradation products of pyrimidines are carbon dioxide and ammonia, which are lost in the general body pools. Only recently has a "pyrimidine disease," orotic aciduria, been described (1). The role of pyrimidine nucleotides in the formation of nucleic acids and in a number of coenzyme functions emphasizes the importance of quantitative data concerning pyrimidine metabolism in man. This report describes studies of the enzymatic steps in normal blood cells leading to the formation of the first pyrimidine product, orotic acid.

EXPERIMENTAL PROCEDURE

Preparation of reactants. Carbamylphosphate (CAP) was synthesized and analyzed as described by Jones, Spector and Lipmann (2). *l*-Carbamylaspartate-C^{14} (CAA-C^{14}) was prepared from l-aspartate and cyanate-C^{14} (3) and measured by the Koritz and Cohen procedure (4). Cyanate-C^{14} was synthesized by the fusion of potassium carbonate and urea-C^{14} (5). *dl*-Dihydroorotate-C^{14} (DHO-C^{14}) was synthesized by the catalytic hydrogenation of orotate-C^{14} (labeled in carbon 6) using a rhodium-alumina catalyst (6) and measured as described by Yates and Pardee (7). Nonlabeled *l*-dihydroorotate was synthesized by the method of Miller, Gordon and Englehardt (8). *dl*-5-Carboxymethylhydantoin-C^{14} (5-CMH-C^{14}) labeled in the carboxyl group was prepared from the above *dl*-DHO-C^{14} (3) and measured as described by Lieberman and Kornberg (9). Nonlabeled 5-CMH was correspondingly prepared from carbamylaspartate. Bicarbonate-C^{14} was prepared from barium carbonate-C^{14} and analyzed as described by Zamecnik, Frantz, Loftfield and Stephenson (10). Uniformly labeled l-aspartate-C^{14} was purchased from Nuclear Chicago Corporation. Urea-C^{14} and orotate-C^{14} were purchased from New England Nuclear Corporation. In enzymatic assays, the above isotopic precursors were added in the following specific activities (infinite thinness, copper or glass planchettes, Nuclear Chicago gas flow counter with micromil window of approximately 32 per cent efficiency):

Labeled compound	Specific activity (cpm per μM)
bicarbonate-C^{14}	9.3×10^5
l-aspartate-C^{14}	9.0×10^4
l-CAA-C^{14}	2.6×10^5
dl-5CMH-C^{14}	1.9×10^6
dl-DHO-C^{14}	4.2×10^5

Triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN), and crystalline alcohol dehydrogenase (ADH) were purchased from the Sigma Chemical Company.

Preparation of blood cells and incubation. Studies of normal levels of each enzymatic activity were carried out on blood cells from laboratory personnel. Isolation of leukocytes was based on a method of Skoog and Beck (11). Approximately 25 ml. of venous blood was drawn into a siliconized[1] syringe containing 1 ml. of 5 per cent EDTA,[2] mixed quickly, and immediately added to a 60 ml. test tube containing 25 ml. of a 3 per cent dextran,[3] 3 per cent dextrose solution in saline at 0° C. The tube was covered with Parafilm and inverted three to four times to insure complete mixing.

1). *Leukocytes.* After one hour of sedimentation in an ice bath the supernatant suspension was transferred to a 50 ml. Lusteroid tube and spun in an International Refrigerated Centrifuge at 2° for seven minutes at $150 \times G$. Following removal of the supernatant, the sediment was resuspended in 10 ml. of ice-cold isotonic KCl containing 0.4 per cent dextrose. A second centrifugation was carried out at 2° for four minutes at $200 \times G$. The washing procedure was then repeated a second time. The leukocyte-erythrocyte sediment was then suspended in KCl-dextrose at a final leukocyte count of approximately 40,000 to 100,000 per mm.^{3}. The leukocyte count was calculated as the average of eight determinations. The isolation procedure tended to concentrate polymorphonuclear cells at the expense of the less dense lymphocytes

* This study was supported by a research grant from the Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

† A preliminary report of this work has been published in abstract form (J. clin. Invest. 1958, **37**, 932).

[1] All glassware, pipettes, and Lusteroid tubes used in the isolation of intact leukocytes were siliconized with Siliclad, Clay-Adams, Inc., New York, N. Y.

[2] Disodium ethylenediaminetetraacetate in saline.

[3] Lot number HH-1, R. K. Laros Co., Bethlehem, Pa.

so that they constituted approximately 80 to 90 per cent of the total leukocytes. The number of contaminating erythrocytes was usually three to six times greater than the leukocytes. Because of the differences found in cellular enzymatic activities (see below) this degree of contamination was not a significant source of error. The leukocytes were disrupted by sonication or homogenization:

a). *Sonication.* The cellular suspension (2 to 4 ml. in a Lusteroid test tube) was subjected to sonication at maximal intensity for exactly 2.5 minutes using a Raytheon 10 kc. Oscillator (model DF 101). This uniform sonication procedure was sufficient to produce complete destruction of the leukocytes and their nuclei. Prolongation of the sonication time led to a progressive loss of enzymatic activity. Because of the uniformly complete cell destruction obtained, sonication was used in all enzyme preparations from leukocytes except in studies pertaining to intracellular enzyme location.

b). *Homogenization.* Due to the difficulty in obtaining complete or uniform destruction of leukocytes (or other free-floating nucleated cells such as Ehrlich ascites tumor cells) by homogenization, the following procedures were used. The leukocytes were suspended in cold distilled water in a 10 ml. homogenization tube (Potter-Elvehjem type, teflon plunger) and allowed to stand at 0° for five minutes to allow for osmotic damage. The contents were then quickly frozen in acetone-dry ice and thawed under cool tap water. An equal volume of 0.5 M sucrose was added to the damaged cells and vigorous homogenization was then carried out for two to three minutes at 0° C. Examination of the homogenate by phase microscopy or Wright's stained smear showed no undamaged cells, many nuclei with surrounding cytoplasmic or membranous shreds, and many naked nuclei.

2). *Erythrocytes.* An aliquot of the original venous blood was used for determination of red cell indexes, where quantitative data were required. The erythrocyte count was taken as the average of eight determinations. Hemoglobin was determined in duplicate photometrically. The original erythrocyte sediment was resuspended in 25 ml. of the cold dextran-dextrose solution and allowed to stand for an additional 30 minutes at 6° C. The supernatant suspension and the superficial layer of erythrocytes were removed by suction. The cells were then washed twice by suspension in cold isotonic KCl-glucose and centrifugation ($400 \times G$, five minutes at 2° C.). An aliquot from the erythrocyte sediment was suspended in isotonic KCl-glucose in a concentration of approximately three million RBC's per $mm.^3$, and duplicate hemoglobin determinations were carried out. Using this value and the previously determined red cell indexes, the actual erythrocyte count was calculated. This indirect determination of the erythrocyte count was necessary because of a variable residual agglutination of the cells following exposure to dextran. The red cell suspensions, virtually free of leukocyte contamination, were then frozen in acetone-dry ice, thawed under a cold water tap and used immediately.

3). *Incubation.* Incubations were carried out in a final volume of 1 ml. in stoppered 10 ml. Erlenmeyer flasks with shaking at 37° C. in a water bath. In the study of soluble aspartate carbamyltransferase from erythrocyte incubations were carried out in 12 ml. centrifuge tubes.

Analytic methods. Each determination of an enzymatic step involved the incorporation of an isotopic precursor of high specific activity into a product which was then isolated and determined by the carrier technique.

1). *Carbamylaspartate (CAA).* The assays of carbamylphosphate synthetase, aspartate carbamyltransferase, 5-carboxymethylhydantoinase and dihydroorotase (see Figure 1) all led to the incorporation of C^{14} into CAA. The assay of dihydroorotase, carried out in conjunction with the assay of dihydroorotic dehydrogenase is described in the next section. For the other enzymes, the flasks were chilled after incubation, and 10 μM of nonlabeled CAA carrier was added. Protein was precipitated with 0.8 N perchloric acid (PCA), centrifuged and the sediment washed with 0.4 N PCA. The combined supernatant solutions were neutralized with concentrated KOH (phenol red) and allowed to stand in an ice bath. The solution was then decanted from the $KClO_4$ onto a Dowex-1-formate ion exchange column (1×12 cm.). Elution was carried out with the 0.05 M sodium formate adjusted to pH 3.2 with formic acid (9). The elution peak, located by spot tests for the ureide group (12), generally began at 23 to 26 column volumes of eluate and was complete at 28 to 33 volumes. Because of this constancy as many as 12 chromatographic separations could be conveniently run in parallel with individual eluate collections only at the time of the anticipated elution. All of the C^{14} precursors were effectively eliminated by this procedure: aspartate, two to six column volumes; DHO, four to nine column volumes; 5-CMH, eight to fifteen column volumes. [With aspartate-C^{14} as a precursor, the neutralized PCA supernatant was first passed through a Dowex 50-H column (1×6 cm.), to remove the bulk of the isotope.] The eluate containing CAA was neutralized with KOH and added to a second Dowex-1-formate column (1×5 cm.). The column was washed, blown dry and elution was carried out with successive 2.0 ml. aliquots of 2 N formic acid. Again the elution pattern was determined by spot test, with the peak concentration generally appearing after 8 to 10 ml. This second chromatographic procedure allowed: *a*) rapid concentration of the large volume of dilute CAA solution from the first chromatography (approximately 50 ml.), *b*) replacement of sodium formate with volatile formic acid prior to plating at infinite thinness, and *c*) only incidentally some further chromatographic purification. Samples from two successive tubes of eluate were analyzed for CAA content and radioactivity to allow duplicate calculations of specific activity. CAA concentration was determined by the method of Koritz and Cohen (4), modified for a final volume of 3.0 ml. in 2.0 N formic acid, and read in a Beckman model DU spectrophotometer at 550 mμ. Radioactivity was determined on 0.5 ml. aliquots of the eluate (containing 0.3 to 0.6 μM CAA).

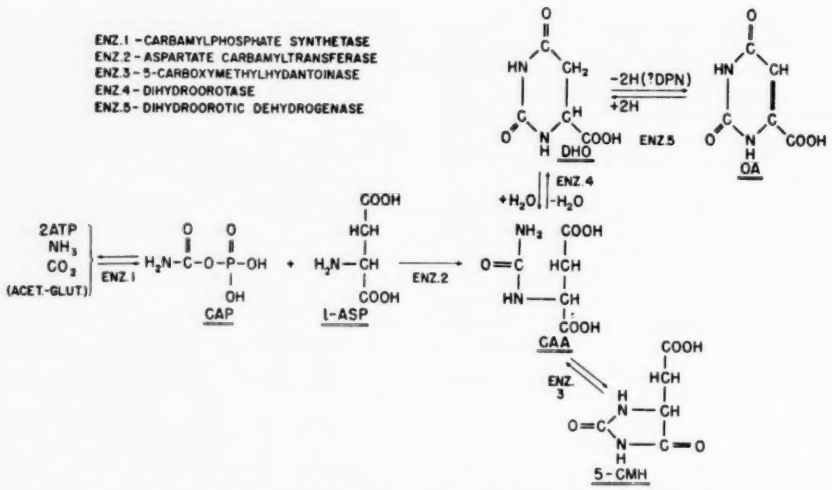


FIG. 1. PATHWAY OF OROTIC ACID BIOSYNTHESIS STUDIED IN HUMAN BLOOD CELLS

Abbreviations include: acet.-glut., acetylglutamate; CAP, carbamylphosphate; l-asp., l-aspartate; CAA, carbamylaspartate; 5-CMH, 5-carboxymethylhydantoin; DHO, dihydroorotate; OA, orotate. Enzymes 1 and 3 were not demonstrated in leukocytes or erythrocytes.

Calculations were based on a total of 5,000 counts per sample using the previously described gas flow counter.

2). Orotic acid (OA) and CAA from dihydroorotate-C^{14} (DHO-C^{14}). The oxidation of DHO-C^{14} to OA and its hydrolysis to CAA (see Figure 1) were studied simultaneously in the same flask. After incubation 5 μM nonlabeled OA carrier and 30 μM CAA carrier were added and proteins were precipitated and washed with PCA as described above. The combined supernatants were passed through a charcoal column (1 × 3 cm. Nuchar Grade C, 40 to 60 mesh) which was then washed with six volumes of water. The charcoal column retained the OA and much of the precursor DHO-C^{14}. The charcoal-column eluate and wash were combined for the isolation of CAA.

a). CAA. The solution containing CAA was then neutralized with KOH with extreme care to pH 7.4 to 7.6 with rapid magnetic stirring and constant monitoring of pH. This extra care was demanded because of the chemical lability of DHO in alkaline solutions (7) causing a nonenzymatic hydrolysis of DHO-C^{14} to CAA-C^{14} to occur at higher pH values (see below). Further purifications and analysis of the CAA were carried out as previously described.

b). OA. The charcoal column was washed with 200 ml. water after which the OA and residual DHO-C^{14} were eluted with 12 ml. of concentrated NH_3-ethanol-H_2O (1:2:2 by volume) onto a Dowex 2-Cl column (1 × 10 cm.). The column was washed with 10 column volumes of 0.03 N HCl to elute DHO-C^{14}. OA was then eluted with 10 ml. of 2 N HCl. The eluate was alkalinized with 6 ml. of 5 N KOH and allowed to stand for 10 minutes to insure conversion of any contaminating DHO-C^{14} to CAA-C^{14} for ease in separation from OA in the subsequent procedures. After being acidified with concentrated HCl the solution was passed through a second charcoal column (1 × 3 cm.), which was washed and eluted as described above into a tapered centrifuge tube. The eluate was taken to dryness under an air jet in a boiling water bath. The residual OA was taken up in hot water and further purified by ascending chromatography on Whatman No. 3 paper with propanol-concentrated formic acid-water (7:1:2) (13). The OA band (R_f approximately 0.40), located by ultraviolet light, was eluted with hot water. Aliquots were taken for counting, as described for CAA, and for measurement by ultraviolet absorption at 280 mμ in 0.1 N HCl. This extensive purification procedure (two charcoal absorptions, one ion exchange chromatography, one paper chromatography) was used because of the low rates of synthesis studied and the consequent low tolerance for C^{14} contamination by the isotopic precursor of high specific activity.

3) *Calculations.* The amount of product synthesized was calculated from the standard carrier formula:

$$\mu\text{M synthesized} = \frac{\mu\text{M carrier added} \times \text{specific activity of isolate (cpm per } \mu\text{M)}}{\text{specific activity of precursor (cpm per } \mu\text{M)}}.$$

When rates of synthesis were to be compared, the results were expressed as millimicromoles (mμM) of product formed per 10^8 WBC or per 10^9 RBC.

RESULTS

Absence of detectable CAA and OA in plasma and blood cells

The carrier technique for recovery and analysis of a product is based on the assumption that the amount of the product in the reaction flask is negligible in comparison with the amount of the added carrier. By the isotope dilution method, therefore, an attempt was made to determine the normal concentrations of CAA and OA in hemic cells. Three μM OA-C^{14} (4,200 cpm per μM) and 5 μM of CAA-C^{14} (4,500 cpm per μM) were added to 8×10^{10} RBC, 8×10^{7} WBC, and 10 ml. spun plasma. The recovered OA-C^{14} and CAA-C^{14} had no change in specific activities. It is estimated that this method would have allowed the detection of 0.3 μM OA and 0.5 μM CAA. "Product dilution" cannot therefore be a significant source of error in the carrier technique employed.

Effect of an internal carrier

In these crude cell preparations the presence of two or more sequential enzymes in the pyrimidine synthetic pathway introduces a source of error, in that during incubation the product of the reaction under study may serve as a substrate for the following enzymatic step. To a large degree this is negated by the choice of experimental conditions (pH, time and so forth) to give biochemical advantage to the preferred reaction and by the low concentration of the product formed. Several attempts were made, however, to determine whether the use of an "internal carrier" during the reaction would serve as a trap and thereby increase the calculated yield of the product. In brief, it was found that a carrier trap during incubation decreased rather than increased the calculated yield. Presumably the tendency toward reversal of the reaction outweighed any trapping function. The carriers were therefore added at the end of incubation in all subsequent experiments.

Carbamylphosphate synthetase

Carbamylphosphate is a labile intermediate which is difficult to purify and analyze in the microquantities anticipated in these cell preparations. The presence of aspartate carbamyltransferase having been demonstrated (see below), attempts were made to detect the presence of CAP-synthetase in hemic cells by coupling the first two reactions shown in Figure 1. In practice the assay depended upon the incorporation of bicarbonate-C^{14} into the ureide carbon of CAA. Conditions similar to those shown to be optimal in rat liver (14) were used in this study with erythrocyte hemolysates and leukocyte sonicates and homogenates. The appearance of C^{14} in CAA could not be demonstrated despite the sensitivity of this system, which would have detected the synthesis of 1 mμM of CAA from bicarbonate-C^{14}. This enzymatic step, even if detectable, would not lend itself to reliable quantitative analysis.

5-Carboxymethylhydantoinase (5-CMH'ase)

This enzyme, which catalyzes the reversible ring closure of CAA to the corresponding hydantoin (see Figure 1), has been described only in *Zymobacterium oroticum* and was found to be absent in rat liver (9). An attempt was made to demonstrate the presence or absence of this enzyme in human blood cells. In two hours of incubation of 0.002 M *dl*-5-CMH-C^{14} (1.9×10^{6} cpm per μM) at pH 8.2 (Tris buffer) with enzyme preparations from leukocytes (*ca.* 1.6×10^{8} cells), erythrocytes (*ca.* 3×10^{9} cells), and rat liver homogenate (*ca.* 1.5 mg. N) no synthesis of CAA could be demonstrated.

Aspartate carbamyltransferase

Aspartate carbamyltransferase, previously studied in rat liver (15) and bacterial systems (16), irreversibly catalyzes the carbamylation of l-aspartate by carbamyl phosphate to yield l-carbamylaspartate, as shown in Figure 1. This reaction also occurs spontaneously in the absence of the specific enzyme so that appropriate nonenzymatic controls must be carried out. Aspartate carbamyltransferase was found to be present in circulating leukocytes and erythrocytes. In hemolysates of erythrocytes the enzyme was largely soluble (in the $100{,}000 \times G$ supernatant). In leukocyte homogenates the activity was confined to the particulate fractions (Table I), with almost half of the activity appearing in the larger cell fragments and nuclei separated at $600 \times G$. More complete fragmentation of the cell particles by sonica-

TABLE I

Location of enzymatic activities in preparations of leukocytes and erythrocytes

Source of enzyme	Aspartate carbamyltransferase CAA formed*	Dihydroorotase CAA formed	Dihydroorotic dehydrogenase OA formed†
	mμM	*mμM*	*mμM*
I. Leukocyte homogenate			
a. Whole homogenate	133	106	5.5
b. 600 × G supernat.	71	54	0
c. 100,000 × G supernat.	1	5	0
II. Leukocyte sonicate			
a. Whole sonicate	370	119	22
b. 600 × G supernat.	294	80	0
c. 100,000 × G supernat.	124	62	0
III. Erythrocyte hemolysate			
a. Whole hemolysate	195	99	0
b. 100,000 × G supernat.	170	84	0

* CAA, carbamylaspartate.
† OA, orotic acid.

tion brought 30 to 40 per cent of the enzymatic activity into solution.

Studies on the characteristics of this enzyme system in man were carried out on erythrocyte hemolysates. These results are summarized by the series of graphs presented in Figure 2. The substrate concentration curves illustrate the high concentration of aspartate (0.01 M) and CAP (0.005 M) necessary for enzyme saturation. The pH curve demonstrated optimal enzymatic synthesis at pH 9.2 to 9.3, with no significant change in nonenzymatic synthesis over the range of pH tested (8.0 to 10.0). Enzymatic activity of aspartate carbamyltransferase varied directly with the number of erythrocytes employed and was linear with time over the first hour of incubation. The increasing rate of nonenzymatic CAA synthesis with time seemed anomalous. It was anticipated that the diminishing concentration of CAP during incubation would result in a corresponding diminution in nonenzymatic synthesis. Based on these studies, standard conditions for assay of asparate carbamyltransferase were established as: 30 minutes incubation, pH 9.0 (Tris buffer 0.2 M); concentration of substrates: aspartate, 0.015 M; CAP, 0.010 M. With these conditions the nonenzymatic control synthesis of CAA was repeatedly found to be 12 to 14 mμM.

Dihydroorotase

Dihydroorotase was studied by hydrolysis of DHO rather than by the ring closure of CAA, allowing a simultaneous determination of dihydroorotase and dihydroorotic dehydrogenase from precursor DHO-C^{14} (see Figure 1). Table I illustrates that dihydroorotase was found in circulating human leukocytes and erythrocytes. In erythrocytes the enzyme was almost completely soluble, but in leukocytes (as with aspartate carbamyltransferase) only a trace of enzymatic activity was brought into solution by homogenization. Approximately 50 per cent of the activity was solubilized by sonication. Table II demonstrates the constancy of CAA synthesis from DHO-C^{14} in a number of variable conditions, which were mostly chosen for study of their possible effect on simultaneous OA synthesis. Mg^{++} has been reported to stimulate the hydrolysis of dihydrouracil but not of dihydrothymine by rat liver hydropyrimidine hydrase, seemingly an analogous enzyme (17). Mg^{++} was without effect on dihydroototase in man.

Some of the biochemical characteristics of dihydroorotase are presented in the graphs of Figure 3. The substrate concentration curve shows enzyme saturation to be attained at approximately

TABLE II

Effect of varying conditions on dihydroorotase activity (CAA formed) and dihydroorotic dehydrogenase activity (OA formed) in a leukocyte sonicate *

Incubation conditions	CAA formed	OA formed
	mμM	*mμM*
Control	79	33
+ 5×10^{-3} M DPN	75	26
+ 5×10^{-3} M TPN	80	23
+ 1×10^{-2} M Mg^{++}	74	16
+ 5×10^{-3} M EDTA	74	29
+ 5×10^{-3} M cysteine	61	36
+ N_2 gas phase		27
+ N_2 gas phase, 5×10^{-3} M DPN, 3×10^{-2} nicotinamide, ADH†		24
Preincubation control	51	31
DPN'ase preincubation‡	56	26

* Abbreviations are as follows: CAA, carbamylaspartate; OA, orotic acid; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate; and ADH, alcohol dehydrogenase.
† Flasks evacuated and flushed with N_2 three times. ADH, approximately 60,000 units.
‡ Preincubation of the enzyme was carried out for 20 minutes at 37° C. with and without a *Neurospora* DPN'ase prior to the addition of precursor DHO-C^{14}.

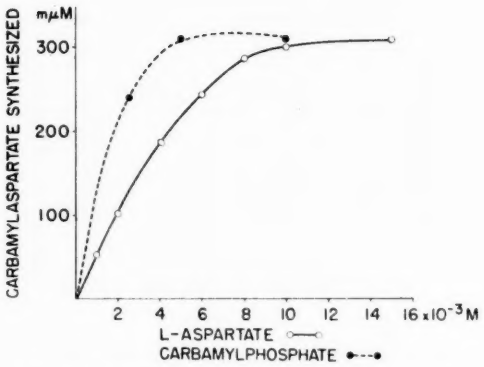


FIG. 2a. SUBSTRATE CONCENTRATION CURVE OF ASPARTATE CARBAMYLTRANSFERASE

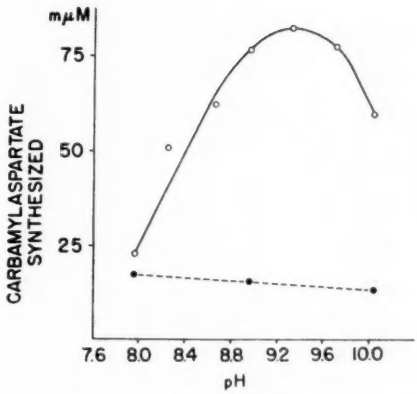


FIG. 2b. pH CURVE OF ASPARTATE CARBAMYLTRANSFERASE

Enzymatic synthesis, ○—○; nonenzymatic synthesis, ●- - -●. Enzymatic synthesis has been corrected for nonenzymatic synthesis.

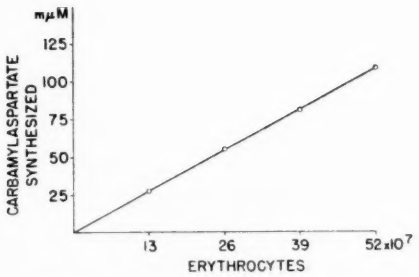


FIG. 2c. ENZYME CONCENTRATION CURVE OF ASPARTATE CARBAMYLTRANSFERASE

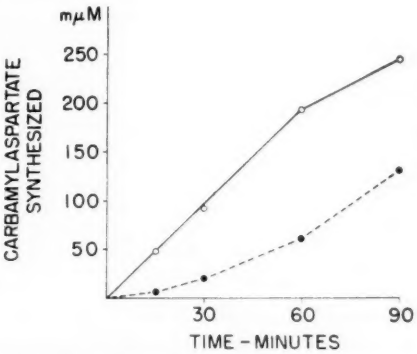


FIG. 2d. TIME RELATIONSHIP OF CAA SYNTHESIS BY ASPARTATE CARBAMYLTRANSFERASE

Enzymatic synthesis, ○—○; nonenzymatic synthesis, ●- - -●. Enzymatic synthesis has been corrected for nonenzymatic synthesis.

1×10^{-3} M DHO. The optimal pH for enzymatic synthesis was 8.2. At pH's greater than 9, nonenzymatic hydrolysis of the hydropyrimidine introduces a significant source of error. Enzymatic synthesis was proportional to enzyme concentration (number of leukocytes) over the range studies, and was linear with time over the first three hours of incubation. Based on these studies and those of dihydroorotic dehydrogenase to be described, the following standard conditions were established for the assay of dihydroorotase: 120 minutes incubation, pH 8.2 (Tris buffer 0.25 M), DHO-C^{14} 10^{-3} M. At pH 8.2 nonenzymatic hydrolysis of DHO-C^{14} was not a source of error (less than one mμM during 120 minutes incubation).

Dihydroorotic dehydrogenase

DHO dehydrogenase was first demonstrated as a DPN requiring enzyme in *Zymobacterium oroticum* by Lieberman and Kornberg (18). DHO dehydrogenase was found in human leukocytes, but not in erythrocytes (Table I). In contrast, nucleated avian erythrocytes did contain this enzymatic activity. A number of attempts to solubilize the enzyme were unsuccessful (sonica-

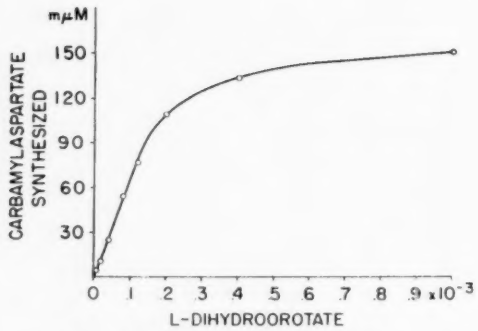


FIG. 3a. SUBSTRATE CONCENTRATION CURVE OF DIHYDROOROTASE

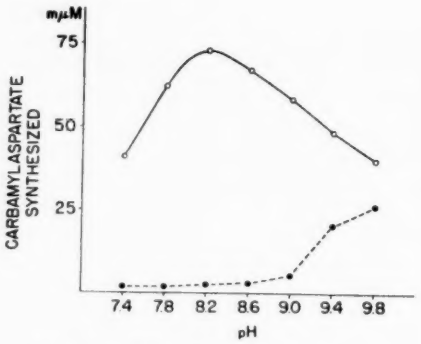


FIG. 3b. pH CURVE OF DIHYDROOROTASE

Enzymatic hydrolysis, ○—○; nonenzymatic hydrolysis, ●- - -●. Enzymatic synthesis has been corrected for nonenzymatic synthesis.

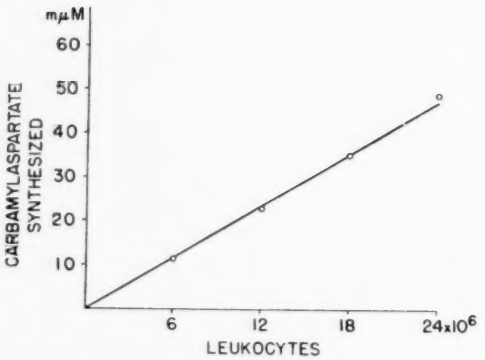


FIG. 3c. ENZYME CONCENTRATION CURVE OF DIHYDROOROTASE

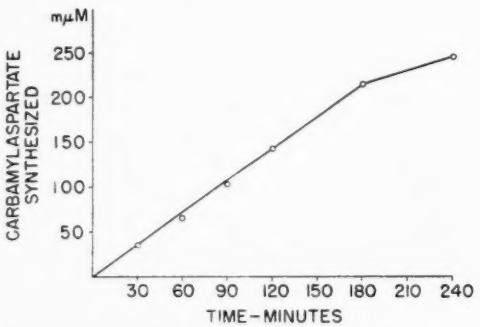


FIG. 3d. TIME RELATIONSHIP OF CAA SYNTHESIS BY DIHYDROOROTASE

tion, freeze-thawing in various buffers, lyophilizing, digitonin extraction), the activity always being sedimented with the nuclei and larger particles at 600 × G. A variety of factors were studied for possible effect on the oxidation of DHO-C^{14} to orotic acid in this system (Table II). With the exception of a slight inhibition by Mg^{++}, no constant effects were obtained. A particular effort was made to demonstrate a requirement for DPN, but such a requirement (reported for bacterial systems) could not be shown. Negative studies included: *1*) Stimulation of activity: addition of 10^{-3} to 10^{-2} M DPN to a dialyzed leukocyte sonicate, use of a DPN regenerating system (alcohol dehydrogenase and acetaldehyde 0.025 M), addition of DPN and the regenerating system to the incubation carried out under nitrogen to diminish any effect of DPN oxidase in the control flask (18), preparation of sonicates or homogenates in nicotinamide (0.03 M), and finally the use of all these conditions simultaneously. *2*) Reduction of activity: On two occasions pre-incubation of the leukocyte sonicate for 20 minutes with a diphosphopyridine nucleotidase (DPNase) prepared from *Neurospora crassa*[4] failed to cause a significant reduction of subsequent orotic acid synthesis from DHO-C^{14}. TPN was also tested, having been shown to be the cofactor for dihydroorotic dehydrogenase from *Corynebacterium* (19), but again no effect could be demonstrated.

Studies on some characteristics of DHO dehydrogenase are summarized in Figure 4. The sub-

[4] We are grateful to Dr. M. N. Swartz for the preparation and purification of this enzyme.

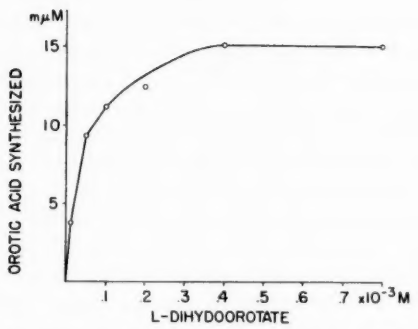


FIG. 4a. SUBSTRATE CONCENTRATION CURVE OF DIHYDROOROTIC DEHYDROGENASE

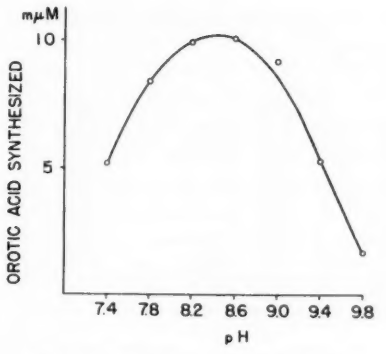


FIG. 4b. pH CURVE OF DIHYDROOROTIC DEHYDROGENASE

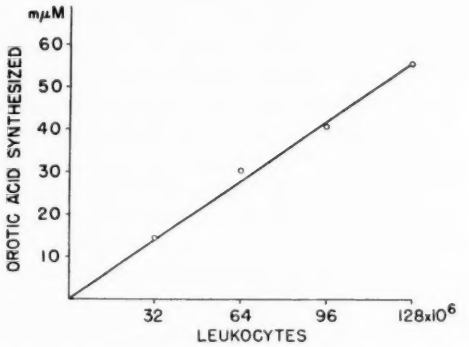


FIG. 4c. ENZYME CONCENTRATION CURVE OF DIHYDROOROTIC DEHYDROGENASE

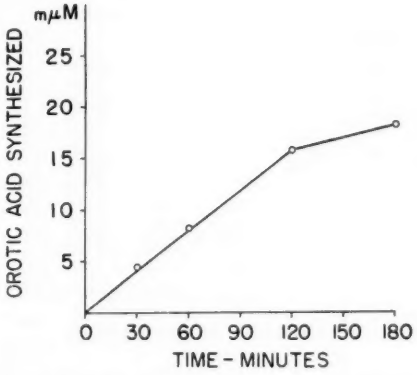


FIG. 4d. TIME RELATIONSHIP OF OA SYNTHESIS BY DIHYDROOROTIC DEHYDROGENASE

strate concentration curve demonstrated maximal enzymatic activity at DHO-C^{14} 5×10^{-4} M. Optimal synthesis occurred at pH 8.4 to 8.6. The formation of orotic acid was directly related to the enzyme concentration (number of sonicated leukocytes) and to the time of incubation for the first 120 minutes. The standard conditions which were subsequently used for the assay of DHO dehydrogenase have been described for dihydroorotase above.

Normal levels of enzymatic activity

A study of the normal levels of these enzymatic activities in mature erythrocytes and leukocytes was carried out with 18 determinations on 12 normal subjects, summarized in Figure 5. Comparative studies with avian erythrocytes illustrate the presence of dihydroorotic dehydrogenase in nucleated erythrocytes, in contrast to the absence of this activity in human erythrocytes. The data in Figure 5 also illustrate that circulating human leukocytes are roughly equivalent to rat liver in aspartate carbamyltransferase and dihydroorotase activities but contain only 2.5 per cent of its dihydroorotic dehydrogenase activity.

DISCUSSION

In this study the biosynthesis of orotic acid in man has been shown to follow the same pathway as that demonstrated in bacteria (9, 18) and in rat liver (20, 21). The single exception is that carbamylphosphate synthetase could not be demonstrated in leukocytes or erythrocytes. CAP was readily shown to be a substrate for the succeeding reaction, the formation of CAA. It is probable that the method employed, that of attempting to

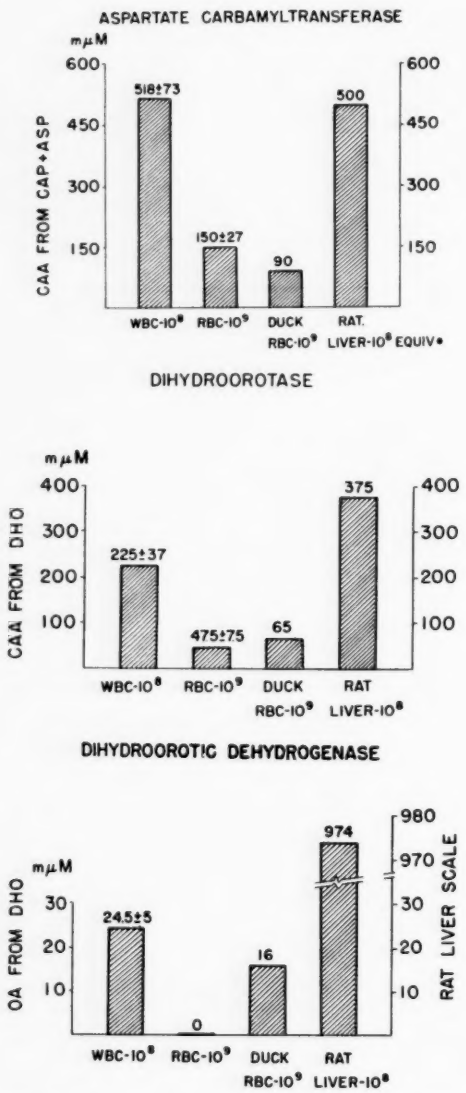


FIG. 5. NORMAL LEVELS OF ENZYMATIC ACTIVITIES IN HUMAN BLOOD CELLS COMPARED WITH DUCK ERYTHROCYTES AND RAT LIVER HOMOGENATE (OF EQUIVALENT N CONTENT AS 10^8 WBC)

"trap" the synthesized CAP as CAA in the presence of aspartate and aspartate carbamyltransferase, was too insensitive to detect the minute amounts of this labile product formed. In mammalian tissues other than the liver, carbamylphosphate synthetase either could not be detected (blood and muscle) or was present at only about one per cent the concentration in the liver (22), where its activity is greatly enhanced by a function in the urea cycle. In these studies, no 5-carboxymethylhydantoinase activity was found in hemic cells or in rat liver. The function of this enzyme, previously demonstrated only in a bacterial system (9), has remained obscure.

Aspartate carbamyltransferase was present as a soluble enzyme in erythrocytes and in the particulate fractions of leukocytes. Similarly, it has been reported to be located in the particulate fractions of rat liver (14). Most studies of this enzyme have been carried out in bacterial systems (16). The enzyme in man resembles the enzyme from *E. coli* in its requirement for high concentrations of l-aspartate (10^{-2} M) and of CAP (5×10^{-3} M) for enzyme saturation, although in the bacterial system an even higher concentration of l-aspartate (7.5×10^{-2} M) is required. The pH optimum in man was found to be 9.0 as opposed to 7.5 in *E. coli*. It is not surprising that such differences are found. A major qualitative difference in bacterial and mammalian carbamylation reactions exists in the requirement of the mammalian system for a substituted glutamate (preferably acetylglutamate) in the synthesis of CAP (23). The formation of CAA from CAP and l-aspartate, if at all reversible, has an equilibrium far in the direction of CAA synthesis, the direction therefore chosen for the present assay procedure. Four other reactions involving CAA have been described: the previously discussed reversible ring closure to 5-CMH (9), irreversible decarboxylation to yield carbamyl β-alanine (24), irreversible decarbamylation to aspartate, carbon dioxide and ammonia (25), and reversible ring closure to dihydroorotate (Figure 1) by dihydroorotase.

Dihydroorotase has not been previously studied as an independent enzyme in a mammalian system, although dihydroorotate was shown to be an intermediate in orotic acid synthesis by rat liver (20). The original description of this enzyme (9) did not include data (pH curve, enzyme concentration curve) which would allow comparison with the present studies in man. Hydropyrimidine hydrase, a seemingly closely analogous enzyme which hydrolyzes dihydrouracil and dihydrothymine to their respective carbamyl compounds (17), also demonstrates pH optima for opening

of the hydropyrimidine ring in the alkaline range (dihydrouracil, pH 10.0; dihydrothymine, pH 8.5; this study, dihydroorotic, pH 8.2). This might be anticipated from the lability of this class of compounds in alkaline solutions.

Dihydroorotic dehydrogenase activity was present in leukocytic nuclei and in nucleated avian erythrocytes, but not in adult human erythrocytes. Although this enzyme has been extensively studied in a bacterial system (18, 26), extension of this work to mammalian tissue has been largely limited to the two-step conversion of precursor carbamylaspartate to orotic acid (21). This study failed to demonstrate a requirement for DPN or TPN, previously reported for bacterial systems (18, 19). Evidence has recently been presented that dihydroorotic dehydrogenase is a flavoprotein from which DPN is a secondary but not obligate electron acceptor (26, 27). Oxygen and methylene blue can serve similarly as electron acceptors from the reduced flavin-enzyme complex. Oxygen was probably the electron acceptor in these leukocyte preparations although a trace contamination of DPN in the insoluble particulate system might allow for the extremely low rates of synthesis measured.

The current studies were undertaken largely to develop enzyme assay techniques to be used in the investigation of pyrimidine metabolism in disease. Figure 5 illustrates that aspartate carbamyltransferase, dihydroorotase and dihydroorotic dehydrogenase can be determined in circulating blood cells with acceptable reproducibility of normal enzyme activities. This reproducibility has been found despite the presence of many potential sources of error in these techniques: inaccuracy of cell counts, standardization of sonication, heterogeneity of the leukocyte population, repeated chromatographic procedures, radioactivity counting errors and colorimetric tests of the isolated carrier. A potentially large source of error in the quantitative application of these techniques to disease lies in the heterogeneity of the leukocyte population. The values which are given here were determined from cell mixtures containing approximately 80 to 90 per cent adult polymorphonuclear cells. Comparable studies on mature lymphocytes and on the effect of cell age on enzymatic activity have not yet been carried out.

Leukocyte aspartate carbamyltransferase and dihydroorotase activities were comparable to those found in an equivalent amount of rat liver sonicate (based on nitrogen content). In contrast, leukocyte dihydroorotic dehydrogenase activity was only 0.025 of that found in rat liver. The reason for this marked discrepancy in the oxidative step is not apparent from the studies at hand. The complete absence of dihydroorotic dehydrogenase activity in adult human erythrocytes and its presence in nucleated avian erythrocytes suggest that the enzyme or an essential unidentified cofactor is lost from the cell with the loss of its nucleus during the maturation sequence unique in the erythrocyte. There is a resulting block of pyrimidine synthesis in the mature erythrocyte at the stage of orotic acid formation. The adult erythrocyte is unique in that it contains no nucleic acids requiring pyrimidine synthesis for regenerative purposes. The present data indicate that pyrimidine nucleotide cofactors, which are found in the erythrocyte (28), cannot be renewed by *de novo* synthesis in the adult cell. The possibility of a "salvage synthesis" of pyrimidine nucleotides from uracil (29) has not been excluded, however. Erythrocyte aspartate carbamyltransferase and dihydroorotase, necessary during intramedullary nucleated life, seem to be vestigial in the mature circulating cell.

The techniques utilized in this study measure the maximal activity of an enzyme (presumably a direct function of its concentration) under optimal conditions after destruction of the cell. These *in vitro* studies cannot fully reflect the *in vivo* control of pyrimidine synthesis, of which the regulation of enzyme concentration is but one part. Additional data are required concerning the steady state concentration of each intermediate and the possible function of feedback control (30) in the intact cell. It is apparent that biochemical studies carried out on normal blood cells can be compared with similar studies on the abnormal cells of a variety of hematological disorders. Of equal interest is the use of blood cells as "representative cells" to reflect the enzymatic defects of inborn errors of metabolism, successfully illustrated, for example, in galactosemia (28). The application of these methods for measuring certain aspects of pyrimidine metabolism to the investigation of disease remains to be demonstrated.

SUMMARY

1. Three enzymes leading to the formation of orotic acid—aspartate carbamyltransferase, dihydroorotase and dihydroorotic dehydrogenase—were found to be present in circulating human leukocytes. Aspartate carbamyltransferase and dihydroorotase were also present in erythrocytes. The intracellular locations and some of the biochemical characteristics of these enzymes were determined.

2. Micro assay procedures were developed which gave reproducible results for normal enzymatic activities of aspartate carbamyltransferase, dihydroorotase and dihydroorotic dehydrogenase in sonicates of mature leukocytes and hemolysates of mature erythrocytes.

3. Carbamylphosphate synthetase and 5-carboxymethylhydantoinase could not be detected in these preparations.

4. Free carbamylaspartate and orotate could not be measured by isotope dilution techniques in plasma, erythrocytes, or leukocytes.

5. The methods described allow a first description in man of qualitative and quantitative aspects of pyrimidine metabolism.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. Paul Zamecnik for his advice and review of this work.

REFERENCES

1. Huguley, C. M., Jr., Bain, J. A., Rivers, S. L., and Scoggins, R. Refractory megaloblastic anemia associated with excretion of orotic acid. Blood. In press.
2. Jones, M. E., Spector, L., and Lipmann, F. Carbamyl phosphate, the carbamyl donor in enzymatic citrulline synthesis. J. Amer. chem. Soc. 1955, **77**, 819.
3. Nyc, J. F., and Mitchell, H. K. Synthesis of orotic acid from aspartic acid. J. Amer. chem. Soc. 1947, **69**, 1382.
4. Koritz, S. B., and Cohen, P. P. Colorimetric determination of carbamylamino acids and related compounds. J. biol. Chem. 1954, **209**, 145.
5. Scattergood, A. Alkali metal cyanates. Inorg. Syntheses 1946, **2**, 86.
6. Green, M., and Cohen, S. S. Studies on the biosynthesis of bacterial and viral pyrimidines. II. Dihydrouracil and dihydrothymine nucleosides. J. biol. Chem. 1957, **225**, 397.
7. Yates, R .A., and Pardee, A. B. Pyrimidine biosynthesis in Escherichia coli. J. biol. Chem. 1956, **221**, 743.
8. Miller, C. S., Gordon, J. T., and Engelhardt, E. L. Dihydroörotic acid. J. Amer. chem Soc. 1953, **75**, 6086.
9. Lieberman, I., and Kornberg, A. Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. II. Dihydroorotic acid, ureidosuccinic acid, and 5-carboxymethylhydantoin. J. biol. Chem. 1954, **207**, 911.
10. Zamecnik, P. C., Frantz, I. D., Jr., Loftfield, R. B., and Stephenson, M. L. Incorporation *in vitro* of radioactive carbon from carboxyl-labeled DL-alanine and glycine into proteins of normal and malignant rat livers. J. biol. Chem. 1948, **175**, 299.
11. Skoog, W. A., and Beck, W. S. Studies on the fibrinogen, dextran, and phytohemagglutinin methods of isolating leukocytes. Blood 1956, **11**, 436.
12. Phillips, D. M. P. Chromatography of the N-carbamoyl-amino acids (hydantoic acids). Biochim. biophys. Acta 1954, **13**, 560.
13. Block, R. J., Durrum, E. L., and Zweig, G. A Manual of Paper Chromatography and Paper Electrophoresis. New York, Academic Press Inc., 1955, p. 212.
14. Reichard, P. The enzymatic synthesis of ureidosuccinic acid in rat liver mitochondria. Acta chem. scand. 1954, **8**, 795.
15. Lowenstein, J. M., and Cohen, P. P. Studies on the mechanism of carbamylaspartic acid synthesis. J. biol. Chem. 1955, **213**, 689.
16. Reichard, P., and Hanshoff, G. Aspartate carbamyl transferase from *Escherichia coli*. Acta chem. scand. 1956, **10**, 548.
17. Wallach, D. P., and Grisolia, S. The purification and properties of hydropyrimidine hydrase. J. biol. Chem. 1957, **226**, 277.
18. Lieberman, I., and Kornberg, A. Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. I. Dihydro-orotic dehydrogenase. Biochim. biophys. Acta 1953, **12**, 223.
19. Reynolds, E. S., Lieberman, I., and Kornberg, A. The metabolism of orotic acid in aerobic bacteria. J. Bact. 1955, **69**, 250.
20. Cooper, C., Wu, R., and Wilson, D. W. Studies of some precursors of pyrimidines. J. biol. Chem. 1955, **216**, 37.
21. Wu, R., and Wilson, D. W. Studies on the biosynthesis of orotic acid. J. biol. Chem. 1956, **223**, 195.
22. Anderson, A., and Jones, M. E. Personal communication.
23. Metzenberg, R. L., Marshall, M., and Cohen, P. P. Carbamyl phosphate synthetase: Studies on the mechanism of action. J. biol. Chem. 1958, **233**, 1560.
24. Grisolia, S., Caravaca, J., Cardoso, S., and Wallach, D. P. Metabolism of dihydropyrimidines and related compounds. Fed. Proc. 1957, **16**, 189.

25. Lieberman, I., and Kornberg, A. Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. III. Ureidosuccinase. J. biol. Chem. 1955, **212**, 909.
26. Graves, J. L., and Vennesland, B. The stereospecific hydrogen exchange in the dihydroorotic dehydrogenase reaction. J. biol. Chem. 1957, **226**, 307.
27. Friedmann, H. C., and Vennesland, B. Purification and properties of dihydroorotic dehydrogenase. J. biol. Chem. 1958, **233**, 1398.
28. Kalckar, H. M., Anderson, E. P., and Isselbacher, K. J. Galactosemia, a congenital defect in a nucleotide transferase. Biochim. biophys. Acta 1956, **20**, 262.
29. Reichard, P., and Sköld, O. Enzymes of uracil metabolism in the Ehrlich ascites tumor and mammalian liver. Biochim. biophys. Acta 1958, **28**, 376.
30. Yates, R. A., and Pardee, A. B. Control of pyrimidine biosynthesis in *Escherichia coli* by a feedback mechanism. J. biol. Chem. 1956, **221**, 757.

STUDIES ON ENHANCED FIBRINOLYTIC ACTIVITY IN MAN *

By SOL SHERRY, ROBERT I. LINDEMEYER, ANTHONY P. FLETCHER AND NORMA ALKJAERSIG

(From the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Mo.)

(Submitted for publication October 15, 1958; accepted January 30, 1959)

Over the years there have been reports describing the occurrence of increased fibrinolytic activity in the blood of both humans and experimental animals in association with a wide variety of circumstances which include wet cupping (1), peptone injections (2), operations (3), emotion (4), exercise (5), adrenaline injections (5) and sudden death (6, 7). In the past decade low levels of fibrinolytic activity have been observed to occur spontaneously in normal, nonstressed adults (8) while significantly increased levels of activity have been noted following acetylcholine injections (9), electroshock (10), circulatory collapse (11), pyrogen reactions (12), local ischemia (13, 14), pulmonary surgery (15) and such obstetrical complications as amniotic fluid embolism, *abruptio placentae* and fetal death *in utero* (16).

The reports mentioned in the preceding paragraph represent only a few of the circumstances in which increased fibrinolytic activity has been observed but illustrate the diverse nature of the clinical and experimental factors that have been reported to produce increased fibrinolytic activity. The mechanism underlying this activity has not been elucidated, but has been variously ascribed to either *1*) a reduction in the antiplasmin level of the blood or *2*) the presence in the blood of an activator of plasminogen.

Recent advances in the basic knowledge of the fibrinolytic enzyme system of human plasma (17, 18) and the development of new assay techniques have offered an opportunity to reinvestigate the occurrence and mechanism of enhanced fibrinolytic activity in patients following various stimuli.

A study was therefore carried out to determine the influence of electroshock, pyrogens, epinephrine, acetylcholine, ischemia and exercise upon the blood fibrinolytic system. It was shown that enhanced fibrinolytic activity occurred in the blood of almost all patients following electroshock, pyrogens and severe exercise and that a less consistent response occurred following epinephrine, acetylcholine and ischemia. Furthermore, it was possible to demonstrate the appearance in the plasma of a plasminogen activator following each of the circumstances mentioned above. Finally, it is suggested on the basis of the data that the enhanced fibrinolytic activity observed following these stimuli was not due to the presence of increased amounts of circulating plasmin but, rather, to the presence of an increased level of circulating activator.

MATERIALS AND METHODS

Biochemical studies

A variety of assay procedures was employed to test most samples. Unless otherwise designated, all blood specimens were collected from an antecubital vein, oxalated and immediately refrigerated in an ice chest until assayed.

1. Assays for fibrinolytic activity. a) Whole blood clot lysis. Whole blood clots were made with 0.9 ml. of oxalated blood and 0.1 ml. of thrombin (Parke-Davis; 10 units per ml. in 0.01 M veronal buffer, pH 7.4). Clots were incubated at 37° in a water bath and the time for complete lysis recorded.

Samples of spontaneously clotted whole blood were compared with oxalated samples clotted with thrombin. In most instances, the former were found to have longer lysis times [1] and for reasons of convenience the latter technique was utilized throughout these experiments.

* This study was supported by grants-in-aid from the National Heart Institute of the National Institutes of Health, United States Public Health Service, and the Lederle Laboratories Division, American Cyanamid Co.

[1] When oxalated blood was clotted with thrombin in the presence of calcium, the lysis time was prolonged as compared to specimens without calcium. An explanation for this difference is suggested by the studies of Medart (19) who demonstrated that in the presence of calcium ions, fibrinogen and fibrin are made more resistant to the action of proteolytic enzymes. Since the submission of this manuscript, similar observations have been reported by Fearnley and Ferguson (Clin. Sci. 1958, 17, 555) and the effect attributed to the inhibitory action of calcium on fibrinolytic activity.

b) *Plasma euglobulin clot lysis.* The plasma euglobulins were precipitated from 0.5 ml. plasma according to the method of Milstone (19 volumes water containing 0.32 volume 1 per cent acetic acid added to 1 volume plasma) (20). After centrifugation, the precipitate was resuspended in 0.5 ml. 0.01 M veronal buffer, pH 7.4, and clotted with 0.5 ml. of thrombin (2 units per ml.). Clots were incubated at 37° C. and the time for complete lysis recorded.

This technique was found to be very sensitive for assaying the small amounts of fibrinolytic activity in plasma and was therefore extensively utilized in these studies. Since with fibrinolytic assays, the activity is a direct function of the reciprocal of the lysis time (17), a logarithmic plot of the lysis time *vs.* units of activity exhibits a linear relationship. Utilizing such a plot, and arbitrarily assigning an activity of 1 unit to a lysis time of 30 minutes, lysis times observed with the plasma euglobulin clot lysis technique were converted into units of fibrinolytic activity. All lysis times greater than 300 minutes are referred to as less than 0.1 unit.[2]

c) *Fearnley clot lysis technique* (*21*). Employing sterile pipettes and working in a cold-room as proscribed by Fearnley, Revill and Tweed (21), 0.5 ml. plasma samples were serially diluted from 1 : 4 to 1 : 32 in 0.1 M veronal buffer in saline, pH 7.4, and were then clotted with 0.5 ml. thrombin (2 units per ml.). Clots were incubated at 37° C. and the time of complete lysis recorded.

This method was found to be more cumbersome and less sensitive than the plasma euglobulin method and possessed the further disadvantage of employing several dilutions of plasma.

d) *Astrup fibrin plate* (*22*). As with previous techniques, this method, which involves the measurement of the area of lysis produced by test solutions placed upon a standard, unheated, bovine fibrin plate, does not distinguish between plasminogen activators and plasmin. It was found to be relatively insensitive for measuring the fibrinolytic activity in whole plasma since incomplete zones of lysis were produced. However the application of the *plasma euglobulin* from the plasma of patients with enhanced fibrinolytic activity frequently produced complete zones of lysis.

e) *I^{131}-Trace-labeled human plasma clots* (*23*). This method involves the measurement of the lysis of preformed human plasma clots, enriched with small quantities of purified human plasminogen and tagged with trace amounts of bovine I^{131}-labeled fibrinogen. The clots, formed upon wire-spirals, were incubated at 37° C. in the test solution (plasma, or euglobulin solution), after which the clots were removed, washed with 0.9 per cent saline, and the radioactivity released into the supernatant determined.

The lysis of these clots has been demonstrated to be directly related to the level of plasminogen activator in the test solution and to be relatively insensitive to the presence of plasmin (23).

II. Demonstration of plasminogen activator in plasma. Activators of plasminogen in plasma were demonstrated by studying the rate of activation of plasminogen in an activation mixture consisting of purified human plasminogen and the patient's plasma euglobulin solution. The human plasminogen was added to make available a uniform excess of substrate. The use of the euglobulin fraction, rather than whole plasma, as the source of activator, obviated the masking effects of plasma inhibitors. Plasminogen was prepared according to the method of Kline (24). Identical 0.2 ml. aliquots of a purified plasminogen preparation containing 9.70 casein units per ml. (25, 26) were incubated at room temperature with 1.8 ml. of resuspended plasma euglobulin in 0.01 M veronal in 0.9 per cent saline, pH 7.4. At intervals during the incubation period, 0.2 ml. aliquots were removed and added to tubes containing 0.7 ml. of 0.3 per cent bovine fibrinogen[3] in buffer, and clotted with 0.1 ml. of buffered thrombin (10 units per ml.). The lysis times of these clots, incubated at 37° C., were then determined and expressed in activity units (as defined for the plasma euglobulin clot lysis technique).

In samples containing an activator of human plasminogen, there was a progressive shortening of the clot lysis time.[4] Figure 1 illustrates the sensitivity of this technique to small amounts of purified urokinase,[5] a known plasminogen activator, and the lack of activation produced by relatively large amounts of spontaneously activated purified human plasmin (28) or in the absence of urokinase (control).

III. Assays for plasma plasminogen, plasmin, antiplasmin and fibrinogen. a) *Plasminogen.* Plasminogen was measured by conversion to plasmin with optimal amounts of streptokinase and assay of the plasmin formed. Two techniques were employed:

[2] Most specimens from "normal" adults will lyse in from 6 to 24 hours by this technique; however, shorter or longer lysis times are not unusual.

[3] Kindly supplied to us by Dr. Kent Miller and containing 92 per cent clottable fibrinogen. The sensitivity of this technique is significantly affected by the purity of the fibrinogen preparation employed. A fibrinolytic assay was employed since the measurement of the small quantities of plasmin formed during the activation required a highly sensitive technique.

[4] In the presence of an activator, but not in its absence, activation of the plasminogen in the plasma euglobulin solution alone could be demonstrated, but the addition of purified human plasminogen, as above, markedly increased the sensisivity of the technique, *e.g.*, in one experiment the fibrinolytic activity increased 1.61 fibrinolytic units in a 30 minute period when the extra plasminogen was present, and only 0.42 unit in its absence. Although a progressive increase in fibrinolytic activity implies the formation of plasmin, similar findings could be obtained if increased amounts of activator were formed.

[5] Kindly supplied by Dr. J. Ploug, Leo Pharmaceutical Co., Copenhagen. The preparation contained 5,100 units per mg. (27).

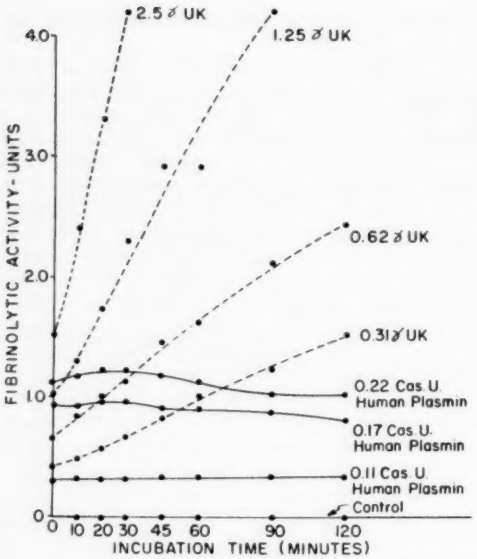


FIG. 1. ACTIVATION CURVES OBTAINED WITH HUMAN PLASMINOGEN PLUS UROKINASE

Varying amounts of urokinase incubated with purified human plasminogen at 25° C. Aliquots removed at time intervals and tested for fibrinolytic activity in a bovine fibrinogen-thrombin clot system. Fibrinolytic activity expressed in fibrinolytic units (see text). No activation occurs with human plasmin alone or in the absence of activator (control). The proteolytic activity of the human plasmin preparations are expressed in casein units (Cas. U.) (25, 26).

1) Casein proteolysis by a modification (25) of the method of Remmert and Cohen (26). In using this assay for plasma plasminogen, advantage was taken of the observation that acidification of human plasma to pH 2.0 destroys a considerable portion of the antiplasmin activity and yields aproximately a threefold increase in proteolytic activity upon the addition of streptokinase (23). Therefore, all plasmas to be assayed by this method first were adjusted to pH 2.0 by the addition of one-sixth N HCl, allowed to stand for 15 minutes, and then reneutralized with one-sixth N NaOH.

2) Hydrolysis of benzoyl-1-arginine methyl ester (BAMe). Based on the study of Schwert and Takenaka (29), a simple spectrophotometric assay for the hydrolysis of BAMe was devised which depends on the absorption difference between benzoyl arginine and BAMe at 2,580 Å. Preliminary observations revealed that the method of Schwert and Takenaka could be readily applied to the assay of plasminogen in plasma and the technique finally developed was as follows[6]:

[6] The detailed observations upon which this technique was developed will form the basis of a separate report.

Two-tenths ml. of 1:5 dilution of plasma in 0.05 M phosphate buffer, pH 7.6, was added to 0.2 ml. of a 6,000 unit per ml. streptokinase solution in buffer. After two minutes incubation at 37° C., 6 ml. of 0.5 mg. per ml. BAMe in buffer was added and a 3 ml. aliquot immediately withdrawn into a tube containing 0.1 ml. 10 per cent acetic acid, which lowers the pH to 5.0 and stops the enzymatic reaction. After incubating the remainder at 37° C. for one-half hour, a second 3 ml. aliquot was withdrawn into 0.1 ml. 10 per cent acetic acid. The optical density of the 30 minute aliquot was read against the zero specimen at 2,580 Å. The spontaneous BAMe esterase activity of the plasma specimen was simultaneously determined by substituting 0.2 ml. buffer for the streptokinase solution in the above method. The plasminogen activity was calculated by subtracting the spontaneous activity from the total activity observed in the streptokinase sample. The results were expressed in units per ml., with one unit representing 1 μM of substrate hydrolyzed per minute under the conditions of the test (30). With this assay, normal plasma contains $65 \pm 15 \times 10^{-3}$ units per ml. plasma.

b) *Plasmin.* Plasmin was assayed by measuring the proteolytic activity of normal and previously acidified plasma on casein. Casein proteolysis was assayed by a modification (25) of the technique of Remmert and Cohen (26).

Observations were also made of the spontaneous BAMe esterase activity of plasma as noted in the previous section. Although the normally observed spontaneous activity cannot be attributed to plasmin alone, since a number of other serum enzymes are also capable of hydrolyzing BAMe, the appearance of increased amounts of plasmin in the circulation would be expected to increase the level of this plasma activity.

c) *Anti-plasmin.* The inhibition of spontaneously activated human plasmin by plasma was used as a measure of anti-plasmin activity. Plasmin was prepared as described by Alkjaersig, Fletcher and Sherry (28). Eight-tenths ml. aliquots of a purified plasmin preparation containing 33.3 casein units per ml. (57 casein units per mg. tyrosine) diluted 1:5 with 0.1 M phosphate buffer, pH 7.6, was incubated at room temperature for 15 minutes with 0.2 ml. phosphate buffer or 0.2 ml. of the plasma to be assayed. The proteolytic activity upon casein was measured and expressed as per cent-inhibition by plasma of the plasmin specimen. This method, although not a precise measure of anti-plasmin, was found to give reproducible results in these studies.

d) *Fibrinogen.* The fibrinogen content of plasma and euglobulin samples was assayed by the method of Ratnoff and Menzie (31).

IV. Inhibition studies with epsilon amino caproic acid. The demonstration that epsilon amino caproic acid is a strong competitive inhibitor in the activation of plasminogen by streptokinase, urokinase and tissue kinases and is a much weaker inhibitor of plasmin (25) afforded an opportunity to investigate whether the enhanced fibrinolytic activity seen in the plasma of patients was due to

the presence of preformed plasmin or to the presence of a plasminogen activator (*i.e.*, with activation of clot plasminogen). Figure 2 cites observations on the effect of a final concentration of 10^{-4} M epsilon amino caproic acid on the fibrinolytic activity observed with an activator alone (urokinase) and with spontaneously activated human plasmin. The test system consisted of 0.5 ml. 0.3 per cent bovine fibrinogen (Armour), 0.1 ml. of urokinase or plasmin, 0.1 ml. 0.001 M epsilon amino caproic acid (0.1 ml. buffer alone used for controls) and 0.3 ml. thrombin, 4 μ per ml. All solutions were made up in 0.01 M veronal buffer, pH 7.4. Note the lack of significant inhibition of plasmin by this concentration of epsilon amino caproic acid and the significant inhibition (approximately 30 per cent) observed with the activator preparation. The test system employed for plasma study was as follows: 0.5 ml. of reconstituted plasma euglobulin solution, 0.1 ml. of buffer or 0.001 M epsilon amino caproic acid; and 0.4 ml. of thrombin, 4 μ per ml. (all solutions in 0.01 M veronal buffer, pH 7.4.). The clots were incubated at 37° C., the lysis time was recorded and expressed in fibrinolytic units.

Experimental studies

Electroshock therapy. Samples were taken from 70 patients undergoing therapeutic electroshock.[7] Patients routinely received premedication with 0.4 mg. atropine and 400 mg. meprobamate. Venous samples were collected just before and within one minute after the convulsion ensuing from electroshock.

Pyrogens. Studies were made on five male patients receiving intravenous pyrogens, four of whom were given 0.2 γ of pyrexal (Wander), and one who received 30 million units of triple typhoid vaccine. Blood specimens were drawn before the injection and at one and two hour intervals afterwards. All patients experienced a temperature elevation of from 1.0 to 2.2° F. (rectal) during the second hour after injection and this elevation was associated with a "chill" in all cases but one.

Epinephrine. Thirteen patients were studied in the following manner: A control venous sample was taken, then 0.5 to 0.6 ml. of 1:1,000 epinephrine was given subcutaneously. Fifteen minutes later a second venous sample was drawn. In most patients, this amount of epinephrine was sufficient to produce an increase in pulse rate of 15 to 25 per minute.

Acetylcholine (AcCh). The effect of 50 mg. of intravenous AcCh was studied in two dogs. After "control" venous samples were drawn, 50 mg. of AcCh in 1 ml. water was rapidly injected. This resulted in hyperpnea, marked salivation, spontaneous defecation and urination within one minute after injection. Venous samples were drawn five minutes after injection.

In an attempt to determine whether acetylcholine may locally stimulate the production of fibrinolytic activity in veins, the following experiment was carried out on 15 patients. After a control venous sample was drawn, blood pressure cuffs were applied above each elbow and inflated to diastolic pressure. One hundred μg. of acetylcholine in 0.1 ml. saline was then injected either into or alongside of an antecubital vein of one arm. Two minutes

[7] At the Malcolm Bliss Mental Health Center. We are indebted to Drs. George Ulett and Kathleen Smith for the opportunity to study these patients.

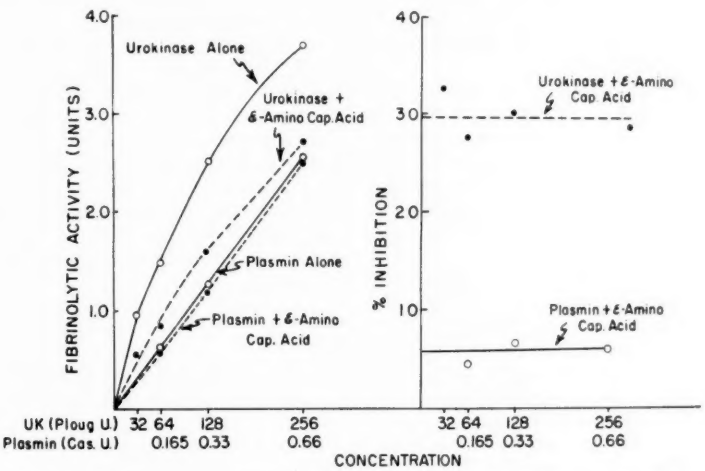


FIG. 2. INHIBITION BY EPSILON AMINO CAPROIC ACID (10^{-4} M) OF THE FIBRINOLYTIC ACTIVITY OBSERVED WITH UROKINASE AND HUMAN PLASMIN IN A BOVINE FIBRINOGEN-THROMBIN CLOT SYSTEM (LEFT PANEL)

Right panel: Results expressed as per cent inhibition of initial activity. Concentrations of urokinase and human plasmin plotted along abscissa.

TABLE I

Results of several fibrinolytic assays, antiplasmin activity and plasma euglobulin fibrinogen concentration immediately before and one minute after electroshock therapy

	Fibrinolytic assays											
	Whole blood clot lysis time		Plasma euglobulin clot lysis time		Fearnley plasma clot lysis*		Lysis of I^{131} trace-labeled human plasma clots by plasma euglobulin		Anti-plasmin activity		Fibrinogen conc. plasma euglobulin	
Patient	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
no.	*hours*		*minutes*				*cpm × 128 in supernatant*		*% plasmin inhibited*		*mg. %*	
21	>24	2	300	30	− − − −	− − − +			69	72	78	57
22	>24	4	>300	50	− − − −	+ + + +			66	56	72	78
23	>24	4	>300	50	− − − −	+ + + +			73	66	85	79
24	>24	4	>300	130	− − − −	+ + + +			59	59	90	94
25	>24	20	>300	50	− − − −	− + + +			60	69	100	90
26	>24	4	>300	45	− − − −	− − − −			66	69	72	67
27	>24	1.5	240	15	− − −	+ + + +			56	66	113	95
28	>24	1	>300	10	− − − −	+ + + +			63	64	102	98
29	>24	>24	>300	240	− − − −	− − − −			65	69	97	110
30	>24	4	>300	50	− − − −	+ + + +			59	60	108	92
31			300	20	− − − −	+ + + +	23.4	232.8				
32			>300	20	− − − −	+ + + +	25.4	170.0				
33			>300	20	− − − −	+ + + +	30.3	164.4				
34			>300	20	− − − −	+ + + +	22.8	198.4				

* + = Complete lysis; − = incomplete lysis. Results are shown for plasma dilutions of 1:4, 1:8, 1:16 and 1:32, respectively. Readings were made after 300 minutes of incubation at 37° C.

later, simultaneous venous samples were drawn from the "injected" vein, a wrist vein of the injected arm, and from an antecubital vein of the opposite arm. The pressure in the cuffs was then released.

Ischemia. Four types of experiments were performed, all on hospitalized patients.

Experiment 1. Venous occlusion alone (10 patients). After a control venous sample was drawn, a blood pressure cuff was applied above the elbow and inflated to diastolic pressure. After five minutes a specimen was taken from the antecubital vein and another, simultaneously, from the opposite, normal arm.

Experiment 2. Arterial occlusion, right arm; venous occlusion, left arm (10 patients). After a control venous sample was drawn, blood pressure cuffs were applied above the elbow to both arms. The right arm was made ischemic by a pressure of 50 mm. Hg above systolic pressure, and the left arm was occluded at diastolic pressure. After five minutes, simultaneous venous samples were drawn from each arm.

Experiments 3. Ischemia at wrist; venous occlusion at elbow (right), and venous occlusion (left) (10 patients). A control venous sample was drawn. The right wrist was then made ischemic with a blood pressure cuff applied at the wrist at a pressure 50 mm. above systolic pressure. Blood pressure cuffs were then placed above the elbow on each arm and inflated to diastolic pressure. After five minutes, simultaneous venous samples were taken from an antecubital vein of each arm.

Experiment 4. Ischemia followed by venous occlusion (10 patients). A control sample was drawn from the left arm. A blood pressure cuff was applied above the right elbow and maintained at 20 mm. Hg greater than systolic pressure for 10 minutes, and then lowered to diastolic pressure for another five minutes. A venous sample was then taken from the right arm and from the opposite, nonoccluded control arm.

Exercise. Studies were made on 12 healthy young adults (nine male, three female) who underwent fairly strenuous exercise (basketball game) over a two hour period. Venous blood specimens were drawn before and at the end of the exercise period. In seven (five males, two females), additional specimens were drawn at the end of 20 minutes of exercise.

Emotion. Nine members of the house staff were subjected to a moderate emotional experience and their fibrinolytic response studied. Prior to the beginning of regular ward rounds by one of us (S. S.), a specimen of blood was collected for "some experimental work." During the conduct of the rounds, the house staff was suddenly exposed to an unsuspected harangue on their inefficiency, ineptitude, and so forth. After 30 minutes of such diatribe, the house staff was informed of the true reason for the experiment and another blood specimen withdrawn.

RESULTS

I. Observations on the production of fibrinolytic activity by various stimuli

a) Electroshock. The results obtained in 14 electroshocked patients utilizing a variety of assay procedures are shown in Table I. Note the relative convenience of the plasma euglobulin clot lysis method for studying changes in plasma fibrinolytic activity. No significant changes were found in the

plasma levels of anti-plasmin. Also, the fibrinogen content of the plasma euglobulin solutions appeared to be independent of the lysis times observed in "before" and "after" samples. Plasma and euglobulin solutions were also tested upon Astrup fibrin plates and although some lysis was produced by most "after" specimens, the areas of lysis were often incomplete and difficult to quantitate, particularly with the plasma samples.

Figure 3A illustrates the pattern of response among 42 patients receiving electroshock, as determined by plasma euglobulin clot lysis, and expressed in activity units. It can be seen that all but four patients had less than 0.1 unit in their "before" samples, whereas only one patient had less than 0.1 unit after electroshock. The average values for "before" and "after" specimens were less than 0.1 unit (*i.e.*, a lysis time greater than five hours) and 0.9 unit (*i.e.*, a lysis time of 35 minutes), respectively.

The age and sex of the patient, and the number of prior shock treatments showed no correlation with the degree of fibrinolytic activity manifested after electroshock. Samples taken from the same patients following electroshock therapy on different days demonstrated the tendency of certain patients' plasma to manifest either consistently fast or slow lysis times. For example, the "after" samples of one patient showed euglobulin clot lysis times of 30, 15, 18 and 21 minutes on four occasions, whereas those of another patient lysed in 150, 50 and 120 minutes on three occasions.

Five patients, whose convulsions were either abolished or greatly minimized by premedication with succinylcholine, all showed very striking fibrinolytic activity after electroshock, demonstrating that the muscular component of electrically induced convulsions was not required for the production of the increased fibrinolytic activity (Figure 3B).

Five patients were given 3.0 mg. atropine sulphate intravenously three to four minutes before electroshock was administered. Blood samples, drawn afterwards in the usual manner, revealed enhanced fibrinolytic activity (Figure 3C), demonstrating that at the time interval and dosage level used, atropine failed to block the appearance of fibrinolytic activity following electroshock.

Five patients receiving electroshock therapy were studied in an effort to determine whether the fibrinolytic activity found after electroshock was locally produced. After control samples were taken, a blood pressure cuff was applied to the patient's right arm and maintained at 50 mm. of Hg

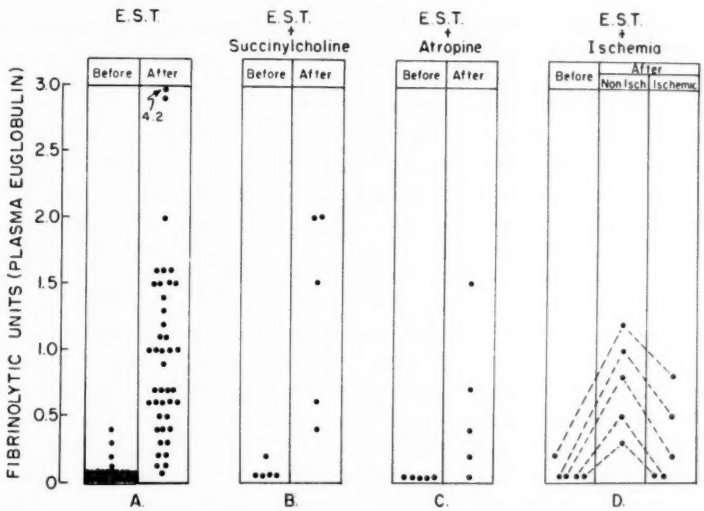


FIG. 3. APPEARANCE OF FIBRINOLYTIC ACTIVITY IN EUGLOBULIN FRACTION OF PLASMA OF PATIENTS SUBJECTED TO ELECTROSHOCK THERAPY

Fibrinolytic activity expressed in units (one unit equivalent to lysis time of 30 minutes under conditions of the test). See text for details.

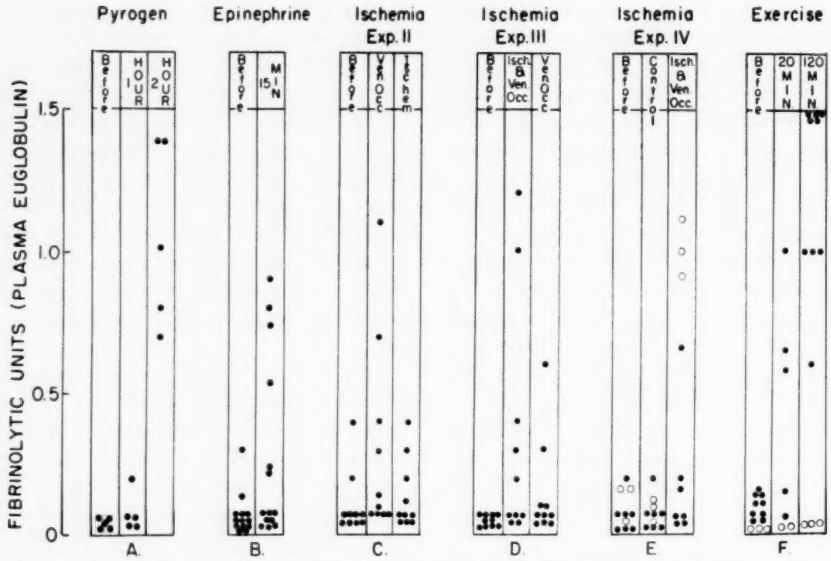


FIG. 4. APPEARANCE OF FIBRINOLYTIC ACTIVITY IN EUGLOBULIN FRACTION OF PATIENTS SUBJECTED TO PYROGENS, EPINEPHRINE, ISCHEMIA AND SEVERE EXERCISE
See text for details.

greater than systolic pressure, thereby arresting the arterial and venous flow in the limb during the convulsion, after which venous samples were taken from the ischemic arm and from the opposite, non-occluded arm. The results shown in Figure 3D reveal that increased fibrinolytic activity was demonstrated in the blood from the ischemic arm of several of the patients, suggesting local production. However, in all cases the activity in the opposite, nonischemic arm was greater. This last observation is consistent with the view that during a period of active ischemia the appearance of fibrinolytic activity may be actually suppressed (14).

b) Pyrogens, exercise, epinephrine and ischemia. Figure 4 cites observations on the fibrinolytic activity induced by pyrogens, exercise, epinephrine and ischemia, as measured by the plasma euglobulin clot lysis technique. Enhanced fibrinolytic activity was observed after each of these procedures, but most consistently and to a greater extent after pyrogens and intense exercise. Accelerated whole blood fibrinolysis and accelerated lysis of I^{131}-trace-labeled plasma clots was observed whenever the plasma euglobulin clot lysis time was rapid and the relative convenience of the plasma euglobulin clot lysis technique was demonstrated again.

With pyrogens (Figure 4A) the enhanced fibrinolytic activity appeared in the second hour specimen or shortly after the "chill." With exercise (Figure 4F) the most marked increase also was evident at the end of the two hour period and specimens drawn at the end of 20 minutes demonstrated a more moderate rise in fibrinolytic activity. The observations, though limited, suggest that males (solid circles) may have a greater response to exercise than do females (open circles).

Epinephrine (Figure 4B) and ischemia produced a less consistent and less striking response but enhancement of fibrinolytic activity was observed in a number of the patients. We were able to confirm the observations of Kwaan and McFadzean (14) that increased fibrinolytic activity does not occur with venous occlusion alone (Experiment 1), but does in association with ischemia where it was demonstrated in occluded venous segments of the opposite arm (Figure 4C), in occluded venous segments of the same arm, proximal to the ischemic area (Figure 4D), and in occluded venous segments draining a previously ischemic area (Figure 4E). Observations made during the latter study suggest that females (open circles) may have a more intense fibrinolytic response to ischemia than do males (solid circles).

c) Acetylcholine. Acetylcholine, when injected intravenously in large amounts into two dogs, produced an increase in circulating fibrinolytic activity. In one animal the activity increased from 0.2 to 1.8 units, and in another from 0.4 to 4.2 units. In the latter animal, whole blood fibrinolysis was complete in the postacetylcholine specimen in 100 minutes. However, 14 of 15 patients who were injected intra- or paravenously with small amounts of acetylcholine showed no increase in fibrinolytic activity and the remaining patient developed a slight activity. The same experiment was carried out using one of the authors (R. I. L.) as the subject, except that a five minute interval between the injection and the drawing of the venous samples was used. In this case, blood from the paravenously injected vein showed 1.2 fibrinolytic units compared to 0.3 unit in the "before" sample. However, no significant activity was found upon three repetitions of this experiment in the same subject.

d) Emotion. Emotional stress, under the conditions of our experiment, failed to elicit a significant increase in circulating fibrinolytic activity in any of the nine subjects studied.

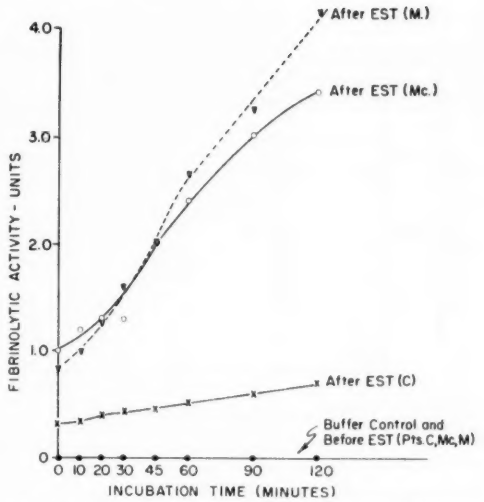


FIG. 5. ACTIVATION CURVES WITH PLASMA EUGLOBULIN OF THREE PATIENTS BEFORE AND AFTER ELECTROSHOCK THERAPY (EST)

Plasma euglobulin incubated with purified human plasminogen at 25° C. Aliquots removed at time intervals and tested for fibrinolytic activity in standard bovine fibrinogen-thrombin clot system. Fibrinolytic activity expressed in units.

II. Demonstration of activator in the plasma of patients with enhanced fibrinolytic activity

Since the lysis of preformed human plasma clots has been shown to be a sensitive index of the level of activator in the test solution and relatively insensitive to the presence of plasmin (23), the marked increase in the lysis of trace-labeled human plasma clots by the "after" specimens of patients

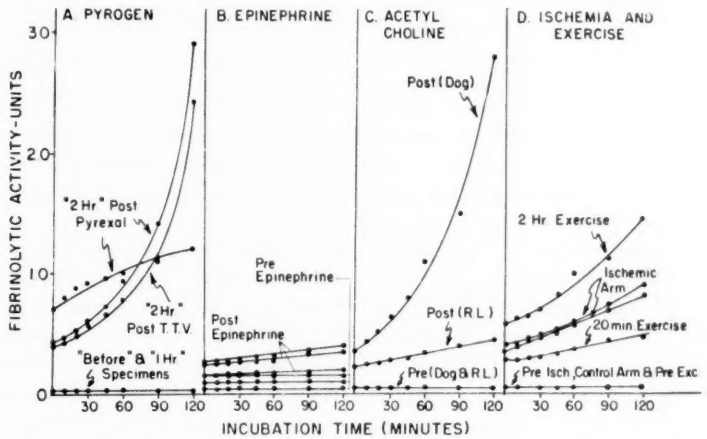


FIG. 6. ACTIVATION CURVES WITH PLASMA EUGLOBULIN OF PATIENTS SUBJECTED TO PYROGENS (A), EPINEPHRINE (B), ACETYLCHOLINE (C), ISCHEMIA AND EXERCISE (D).

Technique as previously described. See text for details.

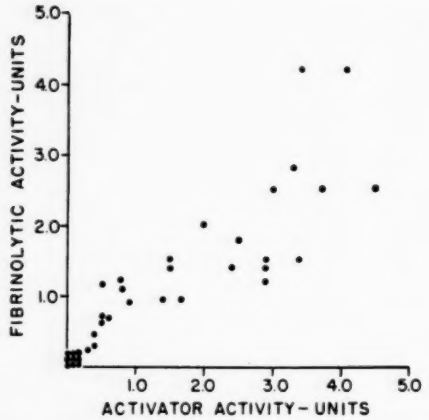


FIG. 7. CORRELATION BETWEEN THE FIBRINOLYTIC ACTIVITY OF THE PLASMA EUGLOBULIN FRACTION AND ITS CONTENT OF ACTIVATOR ACTIVITY

Fibrinolytic activity determined by the plasma euglobulin clot lysis technique. Activator activity determined by activation studies described in text and expressed in arbitrary units (one unit represents that amount of activity required to increase the fibrinolytic activity of the activation mixture by one unit in a two hour incubation period).

undergoing electroshock (Table I, Patients 31 through 34), pyrogens and exercise indicated that the increased fibrinolytic activity was due to the presence of a plasminogen activator. The presence of a plasminogen activator in the plasma of these patients was unequivocally demonstrated by activation experiments utilizing the patients' plasma euglobulin as a source of activator. Figure 5 demonstrates the presence of a plasminogen activator in the euglobulin fraction of the plasma of three patients immediately following electroshock.

Similar observations were made following pyrogens and exercise and in those patients with enhanced fibrinolytic activity following epinephrine, acetylcholine and ischemia (Figure 6).

Precise quantitation of the amount of activator present was not attempted since some of the activation curves (Figure 6A and C) differed from that observed with urokinase, raising considerations requiring further study. However, more activator was present in those patients with the highest fibrinolytic activity, and in general a correlation existed between the amount of activator observed and the fibrinolytic activity determined by the plasma euglobulin clot lysis technique (Figure 7). Thus in specimens with the highest fibrinolytic activity (electroshock, pyrogens and severe exercise) very significant amounts of activator were present whereas with specimens of less intense fibrinolytic activity (epinephrine and ischemia) smaller amounts of activator were present. Figure 6C demonstrates the appearance of a relatively large amount of activator in the plasma of a dog who developed 4.2 units of fibrinolytic activity following 50 mg. of acetylcholine intravenously and a much smaller amount of activator in the plasma of R. I. L. who developed a more moderate increase in fibrinolytic activity (1.2 units) following a paravenous injection of 100 μg. of acetylcholine. No activator could be demonstrated in the plasma specimens of those individuals in whom enhanced fibrinolytic activity did not develop following the various stimuli used.

III. Lack of significant changes in other constituents of the plasminogen system

The plasma levels of fibrinogen, plasminogen and anti-plasmin were studied in a number of patients who developed enhanced fibrinolytic activity following the various stresses described, but no significant changes in these levels as compared to the control observations were noted, nor could any increase in plasmin activity be demonstrated by the techniques employed (casein proteolysis and spontaneous BAMe esterase activity).

IV. Inhibition of enhanced fibrinolytic activity by an inhibitor of plasminogen activation

Since the previous observations suggested that the enhanced fibrinolytic activity was a reflection of the activator concentration rather than any preformed plasmin, this probability was investigated further by means of an activator inhibitor. The plasma euglobulins from nine electroshocked patients were prepared, and the clot lysis time determined in the presence and absence of a 10^{-4} M concentration of epsilon amino caproic acid. The data in Table II demonstrate that the epsilon amino caproic acid exerted a significant inhibitory effect which averaged 39 per cent. This observation, in good agreement with the 30 per cent inhibition observed with urokinase at a similar epsilon amino caproic acid concentration, but significantly

greater than the 4 to 6 per cent noted with plasmin (see Methods section), indicates that the observed fibrinolytic activity reflected the activator concentration rather than preformed plasmin.

DISCUSSION

Enhanced fibrinolytic activity has been demonstrated to occur in the blood of most patients subjected to therapeutic electroshock, pyrogen injections, intense exercise and in dogs given large intravenous doses of acetylcholine. A less consistent fibrinolytic response has been observed to occur among those patients given subcutaneous epinephrine or those subjected to ischemia. No fibrinolytic response was observed after a moderately distasteful emotional experience. The observations of Kwaan, Lo and McFadzean (13), that local paravenous or intravenous acetylcholine consistently produces increased fibrinolytic activity, were not confirmed in these studies.

The mechanism underlying the appearance of marked fibrinolytic activity has been demonstrated, in all the above circumstances, to involve the appearance in the blood of an activator of human plasminogen. Evidence for the presence of a plasminogen activator was furnished both by experiments demonstrating activation of purified human plasminogen and by the results obtained from studies utilizing I^{131}-tagged human plasma clots, the lysis of which has been shown to be a sensitive indicator of the presence of activator in the test solution. Furthermore, the plasma levels of anti-plasmin activity were demonstrated to remain unchanged in samples showing marked fibrinolytic activity. The plasminogen and fibrinogen levels of control and experimental samples, when assayed, were also found to show no consistent change.

The presence of enhanced fibrinolytic activity in plasma need not necessarily be construed as indicating the presence of free plasmin in the circulation. Although fibrinolysis is produced by the action of plasmin, fibrinolytic activity may result either from the presence of preformed plasmin or from the presence of a plasminogen activator which, by virtue of its ability to activate the plasminogen adsorbed onto fibrin during clot formation, also is capable of mediating fibrinolysis. Müllertz (32) recently has reviewed the evidence

TABLE II

Effect of epsilon amino caproic acid on plasma euglobulin clot lysis time following electroshock

Patient	Euglobin clot lysis time		Fibrinolytic activity		Inhibition
	Without EACA	With EACA*	Without EACA	With EACA	
no.	*minutes*		*units*		%
1	63	98	0.49	0.30	38
2	43	70	0.68	0.42	38
3	58	75	0.50	0.39	22
4	45	75	0.66	0.39	41
5	60	115	0.50	0.26	48
6	30	65	1.00	0.46	54
7	30	43	1.00	0.68	32
8	47	85	0.63	0.35	44
9	69	125	0.43	0.27	37

* Epsilon amino caproic acid; 10^{-4} M concentration in test.

which suggests that circulating fibrinolytic activity may be a measure of the presence of plasminogen activator. The observations cited in this article of enhanced fibrinolytic activity occurring in the presence of a plasminogen activator, yet in the absence of demonstrable plasmin or significant changes in the plasminogen or fibrinogen levels, indicate that the increased fibrinolytic activity in our patients was due to the activation of plasminogen after clot formation had occurred rather than to increased levels of circulating plasmin. Additional support for this view is derived from the studies with epsilon amino caproic acid (an inhibitor of plasminogen activation) in which inhibition of the enhanced fibrinolytic activity following electroshock was observed. The findings of Schultz, Moorman, Matoush and Lincoln (33), who were unable to demonstrate increased levels of plasma esterase activity for tosyl arginine methyl ester and lysine ethyl ester in bloods with marked fibrinolytic activity obtained from patients who had received pyrogen injections, are also consistent with this interpretation.

The view that increased fibrinolytic activity is associated with the presence of a plasminogen activator *per se* is also in accord with the evidence recently accumulated by Sherry, Fletcher and Alkjaersig (34) concerning the mechanism of thrombolysis *in vivo*. Under this scheme, the circulating level of plasminogen activator rather than the level of free plasmin is the critical factor determining thrombolysis. Small amounts of activator in the circulation ordinarily result in no measur-

able free plasmin because of the strong anti-plasmin activity of plasma. However, this same amount of activator will produce thrombolysis and to explain this observation it has been suggested that the activator has the ability to diffuse into or adsorb onto the clot and activate the intrinsic clot plasminogen in a relatively inhibitor-free environment. The failure to observe a decrease in plasminogen in plasmas containing an activator may be attributed either to the insensitivity of the assay methods to small changes in plasminogen levels, or to the existence in the circulation of a physiologic mechanism for inhibiting the activator (a mechanism not operative inside the clot). The demonstrated "lability" of the activator in plasma lends some credence to the presence of an inhibitory mechanism.

The demonstration that a plasminogen activator appears in the blood of patients following a wide variety of stimuli (electroshock, pyrogen, exercise, ischemia, epinephrine and acetylcholine) extends the observations of others who have noted the appearance of an activator under select situations. Thus, Müllertz (35) demonstrated the presence of an activator in the spontaneously lytic blood obtained from human subjects after death from anoxia and in patients following electroshock. Albrechtsen, Storm and Trolle (16) describe the presence of both free plasmin and an activator of plasminogen in the blood of a woman dying with amniotic fluid embolism; and Von Kaulla (12), from his studies, considers that the fibrinolytic activity seen after pyrogen injections is most likely the result of the release from the tissues of an activator of plasminogen. The presence of some fibrinolytic activity in all blood specimens has been known for a long time and this phenomenon may be magnified by diluting the plasma (8) or by utilizing the plasma euglobulin clot lysis technique. These observations suggest the possibility that there are trace levels of activator present in the circulation under normal conditions.

Although our studies indicate the significance of activator under physiological circumstances, they should not be construed as indicating that all changes in fibrinolytic activity reflect changes in activator concentration alone. Changes in plasma inhibitors may influence the level of fibrinolytic activity observed. Thus it has been suggested that bishydroxycoumarin and its derivative decrease fibrinolytic activity by increasing the anti-plasmin concentration (36), though it is not apparent from the methods used whether this decrease is due to an increase in anti-plasmin or to an activator inhibitor. Furthermore, the release of large amounts of activator particularly in the presence of reduced plasma inhibition may account for those instances where increased fibrinolytic activity has been associated with changes in fibrinogen, plasminogen and other components of the coagulation mechanism.

Little is known of the nature of the plasminogen activator found in these studies. It is apparently adsorbed onto fibrin during the process of clotting and can be precipitated with the plasma euglobulin fraction. Its lability is suggested by the slowly decreasing fibrinolytic activity of plasma samples stored at $-20°$ C. and its more rapid disappearance at higher temperatures (21, 37). It is an attractive hypothesis that the activator observed in the above studies is the same material in all cases and like other plasminogen activators (38) will prove to be a proteolytic enzyme capable of splitting arginine and lysine esters, but we have no proof for this.

The possibility that a neurogenic mechanism might mediate the production of fibrinolytic activity has been suggested by Kwaan, Lo and McFadzean (13), and is supported by certain of the experimental data in this report. The sudden, local production of fibrinolytic activity in an arm in which the arterial and venous flow had been arrested during electroshock suggests that this response may have been mediated through the nervous system. It is well recognized that patients undergoing electroshock experience a generalized autonomic discharge. Considering the magnitude of this discharge, the failure of atropine as employed in these experiments to block the response to electroshock does not preclude the possibility of the existence of a cholinergic effector-mechanism. Furthermore, both the demonstration of fibrinolytic activity following intravenous acetylcholine in the dog and the similarity of the associated autonomic response to that manifested by patients receiving electroshock, suggest that a cholinergic discharge may mediate the fibrinolytic effect seen with an electroshock. Our ability to confirm the observation of reflex stimulation of fibrinolytic activity (see Ischemia, Experiment 2)

lends additional support to the presence of a neurogenic mechanism.

It has been suggested that fibrinolytic activity is imparted to the blood by the walls of veins (13) since blood withdrawn from superficial veins of an occluded arm may, after various stimuli, contain enhanced fibrinolytic activity. However, in studies currently in progress here employing isotopically-labeled albumin, it has been shown that such "venous" blood consists, in a large degree, of blood recently arrived from the arterial tree; and that it is, therefore, primarily not blood that has been contained within the veins during the period of occlusion. Moreover, data obtained recently from related experiments with ischemia have suggested that, following a period of ischemia, the greatest concentration of fibrinolytic activity is to be found in the fresh blood that perfuses the ischemic limb upon release of the cuff. These findings are consistent with the view that the capillary bed may even be a more important site from which fibrinolytic activity is imparted to the blood.

Although Macfarlane and Biggs have stressed the importance of emotional stress in stimulating fibrinolytic activity (4), it is of interest that not all emotional stress is accompanied by increased fibrinolytic activity. The stress utilized in our study, though real, was neither frightening nor life threatening and, for this reason, may have failed to elicit an increase in activity.

Evidence is accumulating that fibrinolysis is a fundamental physiologic process and not merely a phenomenon limited to certain pathologic states. Fearnley and Lackner (8) have reported low levels of fibrinolytic activity in some healthy, nonstressed adults and we have observed the presence of small amounts of fibrinolytic activity in a number of our patients' "control" blood specimens. In recent articles, Astrup (39) and Duguid (40) have speculated upon the possible relationship of fibrinolysis to the development of atherosclerosis. It is of interest, in this respect, that our female patients appeared to produce a greater fibrinolytic response to ischemia than did males (see Ischemia, Experiment 4.).

SUMMARY

1. Enhanced fibrinolytic activity has been demonstrated to occur in the blood of almost all patients subjected to electroshock and pyrogen injections and in male subjects following severe exercise. A less consistent response has been shown to occur following epinephrine, acetylcholine and ischemia. The plasma euglobulin clot lysis time appears to be a simple and sensitive technique for assaying plasma fibrinolytic activity.

2. The mechanism underlying the appearance of enhanced fibrinolytic activity under the circumstances described has been demonstrated to involve the appearance in the blood of a plasminogen activator.

3. Detailed biochemical studies on plasma of these patients failed to reveal evidence of increased amounts of plasmin or decreased plasminogen or anti-plasmin levels. It is suggested on the basis of the evidence presented that the enhanced fibrinolytic activity was produced by the direct activation of intrinsic clot plasminogen rather than increased amounts of circulating plasmin.

4. Evidence is presented which suggests that plasminogen activators may be locally produced by a neurogenic mechanism and the possibility that the capillary bed may be an important source is discussed.

ACKNOWLEDGMENTS

We are indebted to Doctors Frank Smyrniotis and Arlan Cohn for their clinical assistance and to Miss Geraldine Nelson and Mr. Charles Chisenhall for their technical assistance.

REFERENCES

1. Macfarlane, R. G., and Biggs, R. Fibrinolysis. Its mechanism and significance. Blood 1948, **3**, 1167.
2. Nolf, P. Contribution à l'étude de la coagulation du sang, la fibrinolyse. Arch. int. Physiol. 1908, **6**, 306.
3. Macfarlane, R. G. Fibrinolysis following operation. Lancet 1937, **1**, 10.
4. Macfarlane, R. G., and Biggs, R. Observations on fibrinolysis. Spontaneous activity associated with surgical operations, trauma, etc. Lancet 1946, **2**, 862.
5. Biggs, R., Macfarlane, R. G., and Pilling, J. Observations on fibrinolysis. Experimental activity produced by exercise or adrenaline. Lancet 1947, **1**, 402.
6. Yudin, S. S. Transfusion of cadaver blood. J. Amer. med. Ass. 1936, **106**, 997.
7. Mole, R. H. Fibrinolysin and the fluidity of the blood post-mortem. J. Path. Bact. 1948, **60**, 413.
8. Fearnley, G. R., and Lackner, R. The fibrinolytic activity of normal blood. Brit. J. Haemat. 1955, **1**, 189.

9. Soulier, J. P., and Koupernik, C. Constance de la fibrinolyse au cours du choc acétyl-cholinique. Sang 1948, **19**, 362.
10. Fantl, P., and Simon, S. E. Fibrinolysis following electrically induced convulsions. Aust. J. exp. Biol. med. Sci. 1948, **26**, 521.
11. Tagnon, H. J., Levenson, S. M., Davidson, C. S., and Taylor, F. H. L. The occurrence of fibrinolysis in shock, with observations on the prothrombin time and the plasma fibrinogen during hemorrhagic shock. Amer. J. med. Sci. 1946, **211**, 88.
12. Von Kaulla, K. N. Intravenous protein-free pyrogen. A powerful fibrinolytic agent in man. Circulation 1958, **17**, 187.
13. Kwaan, H. C., Lo, R., and McFadzean, A. J. S. On the production of plasma fibrinolytic activity within veins. Clin. Sci. 1957, **16**, 241.
14. Kwaan, H. C., and McFadzean, A. J. S. On plasma fibrinolytic activity induced by ischaemia. Clin. Sci. 1956, **15**, 245.
15. Kwaan, H. C., McFadzean, A. J. S., and Cook, J. On plasma fibrinolytic activity in cryptogenic splenomegaly. Scot. med. J. 1957, **2**, 137.
16. Albrechtsen, O. K., Storm, O., and Trolle, D. Fibrinolytic activity in the circulating blood following amniotic fluid infusion. Acta haemat. (Basel) 1955, **14**, 309.
17. Sherry, S., and Alkjaersig, N. Studies on the fibrinolytic enzyme of human plasma. Thromb. Diath. Haem. 1957, **1**, 264.
18. Astrup, T. Fibrinolysis in the organism. Blood 1956, **11**, 781.
19. Medart, W. S. Effect of divalent cations on proteolysis of fibrinogen. Proc. Soc. exp. Biol. (N. Y.) 1958, **97**, 728.
20. Milstone, H. A factor in normal human blood which participates in streptococcal fibrinolysis. J. Immunol. 1941, **42**, 109.
21. Fearnley, G. R., Revill, R., and Tweed, J. M. Observations on the inactivation of fibrinolytic activity in shed blood. Clin. Sci. 1952, **11**, 309.
22. Astrup, T., and Müllertz, S. The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. 1952, **40**, 346.
23. Alkjaersig, N., Fletcher, A. P., and Sherry, S. The mechanism of clot dissolution by plasmin. J. clin. Invest. **In press.**
24. Kline, D. L. The purification and crystallization of plasminogen (profibrinolysin). J. biol. Chem. 1953, **204**, 949.
25. Alkjaersig, N., Fletcher, A. P., and Sherry, S. Epsilon amino caproic acid, an inhibitor of plasminogen activation. J. biol. Chem. In press.
26. Remmert, L. F., and Cohen, P. P. Partial purification and properties of a proteolytic enzyme of human serum. J. biol. Chem. 1949, **181**, 431.
27. Ploug, J., and Kjeldgaard, N. O. Urokinase. An activator of plasminogen from human urine. I. Isolation and properties. Biochim. biophys. Acta 1957, **24**, 278.
28. Alkjaersig, N., Fletcher, A. P., and Sherry, S. The activation of human plasminogen. I. Spontaneous activation in glycerol. J. biol. Chem. 1958, **233**, 81.
29. Schwert, G. W., and Takenaka, Y. Spectrophotometric determination of trypsin and chymotrypsin. Biochim. biophys. Acta 1955, **16**, 570.
30. Committee on Biological Chemistry of the Division of Chemistry and Chemical Technology, National Academy of Sciences—National Research Council. International unit of enzyme activity. Science 1958, **128**, 19.
31. Ratnoff, O. D., and Menzie, C. A new method for the determination of fibrinogen in small samples of plasma. J. Lab. clin. Med. 1951, **37**, 316.
32. Müllertz, S. Activation of plasminogen. Ann. N. Y. Acad. Sci. 1957, **68** (1), 38.
33. Schultz, R. L., Moorman, J. A., Matoush, L. O., and Lincoln, A. F. Evaluation of synthetic substrate methods for clinical determinations of fibrinolysis. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 198.
34. Sherry, S., Fletcher, A. P., and Alkjaersig, N. The fibrinolysin system: Some physiological considerations *in* Connective Tissue, and Atherosclerosis, I. Page, Ed. New York, Academic Press Inc., 1959. In press.
35. Müllertz, S. A plasminogen activator in spontaneously active human blood. Proc. Soc. exp. Biol. (N. Y.) 1953, **82**, 291.
36. Niewiarowski, M., and Wegrzynowicz, Z. Der Einfluss von Dikumarol-derivaten auf das fibrinolytische System im Plasma. Abstracts of communications, Fourth International Congress of Biochemistry, Vienna, 1958, p. 163.
37. Truelove, S. C. The lability of human fibrinolysin. Clin. Sci. 1953, **12**, 75.
38. Alkjaersig, N., Fletcher, A. P., and Sherry, S. The activation of human plasminogen. II. A kinetic study of activation with trypsin, urokinase, and streptokinase. J. biol. Chem. 1958, **233**, 86.
39. Astrup, T. The biological significance of fibrinolysis. Lancet 1956, **271**, 565.
40. Duguid, J. B. A concept of the pathogenesis of atherosclerosis *in* Connective Tissue, and Atherosclerosis, I. Page, Ed. New York, Academic Press Inc., 1959. In press.

STUDIES IN KERNICTERUS. I. THE PROTEIN BINDING OF BILIRUBIN *

By GERARD B. ODELL

(From the Harriet Lane Home of The Johns Hopkins Hospital and from the Departments of Pediatrics, Johns Hopkins University School of Medicine and Sinai Hospital of Baltimore, Baltimore, Md.)

(Submitted for publication October 14, 1958; accepted January 15, 1959)

The yellow staining of basal ganglia (kernicterus) in some jaundiced newborn infants has been recognized for many years (1). The association between serum bilirubin concentration and kernicterus (2, 3), the isolation of bilirubin from kernicteric brains (4, 5), and the toxic effect of bilirubin *in vitro* upon oxygen uptake (6) and oxidative phosphorylation (7) are further evidence of a relationship between bilirubinemia and kernicterus. Plasma concentrations of bilirubin exceeding 20 mg. per cent are considered levels above which kernicterus occurs, although many exceptions exist (8, 9), particularly in the premature infant (10). Of particular importance was the observation (11) that kernicterus occurred more frequently in premature infants given sulfisoxazole than in control infants despite lower plasma bilirubin concentrations (12). Animal studies have confirmed these findings (13). Because kernicterus can occur at bilirubin concentrations considerably below 20 mg. per cent and fail to occur at higher concentrations, a study of factors controlling distribution of bilirubin seemed indicated.[1]

The binding of bilirubin to serum proteins has been demonstrated by many techniques (14–20). *In vitro* separation of bilirubin by ultrafiltration from serum protein in detectable amounts has been singularly unsuccessful (20–25). Bilirubin did not appear in ultrafiltrates of plasma unless the concentrations were greater than 80 mg. per cent (22, 25). However, bilirubin gains access to cell cytoplasm at considerably lower concentrations *in vivo*, and probably has to dissociate from serum protein in order to diffuse through cell membranes.

The study of protein-binding of bilirubin by various techniques has led to different interpretations as to which of the serum proteins are primarily involved in the *in vivo* transport of this pigment (17, 25, 26). Alpha 1-globulin, α 2-globulin, iron-binding globulin and β-globulin have all been demonstrated capable of binding bilirubin. All studies are in agreement that serum albumin is concerned in the binding of bilirubin but there is disagreement that albumin binds unconjugated bilirubin (26). Alpha 1-globulin has been shown to bind more unconjugated bilirubin on a weight basis than albumin, but serum contains so little of this component that the amount of bilirubin bound by α_1-globulin is small compared to the total bound bilirubin in very icteric sera (19, 25).

The affinity of albumin for many organic anions is well known and can often be expressed stoichiometrically (27). Methods have been developed for quantitative and qualitative determination of albumin in serum based on this property (28–31). However, many of these methods gave erroneously low results in the presence of bilirubinemia. These latter observations demonstrated that bilirubin would interfere with organic anion binding of serum albumin, presumably by competition for the same loci (29).

The present study reports observations of the effects on the protein binding of bilirubin of certain organic anions some of which are used in clinical medicine. A preliminary report of these studies has been previously presented (32).

METHODS

Sera from 20 newborn infants with high bilirubin concentrations ranging from 10 to 28 mg. per cent were collected and, if not immediately used, were kept refrigerated at 4° C. The causes of the bilirubinemia were erythro-

* These studies have been supported by a grant (H2727) from The National Heart Institute of the National Institute of Health, United States Public Health Service, and gifts from the Pierce Butler Fund for the Study of the Etiology of Cerebral Palsy and Related Disorders.

[1] Suggested to the author by Dr. R. E. Cooke.

blastosis or nonhemolytic hyperbilirubinemia of unknown cause. The sera used contained less than 2 mg. per cent direct reacting bilirubin.

A model serum was employed using dilutions of bovine albumin [2] in a 0.1 M phosphate buffer, pH 7.4. To the diluted albumin solutions, various amounts of aqueous bilirubin were added. The bilirubin solution was made by dissolving crystalline bilirubin (Hoffman-La Roche) in deaerated N/20 sodium hydroxide containing ascorbic acid. The model sera were freshly made on the day of use.

Spectrophotometric studies. Aqueous bilirubin is known to have an absorption maximum between 420 and 440 mμ, and protein-bound bilirubin in aqueous solutions has an absorption maximum in the vicinity of 460 mμ. The molecular extinction coefficients of the two forms of bilirubin are similar and their solutions obey Beer's Law (15, 19, 25).

In the present experiments the effects of organic anions were studied by observing the changes in spectral absorption of bilirubinemic sera or model sera with a Beckman DU model spectrophotometer. The sera from the patients were diluted 20- or 50-fold with water or 0.1 M phosphate buffer, pH 7.4. Equal volumes were pipetted into each of three 1 cm. corex cuvettes. Water was used as a blank. The spectral absorption of the samples was measured between 400 and 500 mμ and the wave length at which maximum absorption occurred in the 460 mμ range was considered that due to protein-bound bilirubin. Subsequently equal volumes of various organic anions in concentrated solutions were pipetted into two of the cuvette samples. A control consisted of an equal dilution of the third sample with water or the phosphate buffer. The additions were made serially with calibrated 20 lambda washout pipettes. The optical density of each solution was measured at the original 460 mμ maximum for the serum after each addition of organic anion or diluent. The spectral curves between 400 and 500 mμ were repeated after the final additions. Decrease in absorption after anion addition greater than that which occurred in the control samples was considered as an indication of uncoupling of bilirubin from protein. The changes in optical density were expressed as percentage changes from the control sample:

$$\% \ \Delta O.D._{460\,max.} = \frac{\epsilon_c - \epsilon_a}{\epsilon_c} \times 100.$$

ϵ_c and ϵ_a are, respectively the optical densities at the absorption maximum of the control sample and the sample to which anion had been added. The contribution of the uncoupled bilirubin to the total absorption at the 460 mμ maximum was not subtracted. Consequently the per cent Δ O.D. represents a minimum value for the uncoupling of protein-bound bilirubin. The pH of the serum samples was measured with a Beckman model G pH meter before and after anion additions in 10 instances: five when water was used as the diluent and five when the phosphate buffer was used. No fall in pH occurred except where mentioned in the results.

Total bilirubin concentrations were measured on the cuvette samples by the Malloy and Evelyn modification of the van den Bergh reaction (33) in the 10 instances where the samples were diluted with water. A Bausch and Lomb Spectronic 20 colorimeter adapted for 1 cm. corex cells was employed.

The concentration of the protein in the bovine albumin model sera was 60 to 240 mg. per cent and was determined from the dilution of the known 30 per cent stock solution. The protein concentrations were not determined in the patient's sera.

Changes in optical density of 0.002 unit were considered significant after individual anion additions when the optical density of the sample was between 0.1 and 0.5 and 0.005 unit when between 0.5 and 1.0.

Bilirubin values determined by the van den Bergh reaction in this laboratory have a 3 per cent error in bilirubin concentrations of 10 mg. per cent or more and a 4 to 5 per cent error in the 5 to 10 mg. per cent range.

Other spectrophotometric analyses were done by quantitative dilution of serum aliquots in calibrated volumetric flasks. The diluent was either distilled water or phosphate-buffer containing various concentrations of organic anions. The spectral analyses were done immediately after dilution or deliberately delayed for various intervals.

The organic anions used were sulfisoxazole diethanolamine (Hoffman La Roche, Inc.); U. S. P. sodium sulfadiazine (Abbott & Co.); sodium salicylate (Baker Chemical Co.); heparin sodium, 10 mg. per 1 ml. (The Upjohn Co.); sulfanilic acid (Merck); glucuronic acid (California Foundation for Biochemical Research); and sodium glucuronate (California Foundation for Biochemical Research).

Ultrafiltration studies. Ultrafiltrations of whole or diluted human and model sera were done by centrifugation at room temperature of sera contained in a dialysis bag suspended in a centrifuge tube.

Various volumes of concentrated sodium salicylate, sodium sulfadiazine, sulfisoxazole and sulfanilic acid were added to the sera to yield concentrations of 5 to 25 mg. per cent before ultrafiltration. A control consisted of a simultaneous ultrafiltration of a serum sample diluted with water equal to the volume of added organic anion solution. Ultrafiltrations were also done with serum samples containing added glucuronic acid and sodium glucuronate in concentrations of 20, 50 and 200 mg. per cent. The ultrafiltration of serum by centrifugation required 10 to 30 minutes.

The qualitative analysis of the ultrafiltrates for bilirubin was done spectrophotometrically and colorimetrically by the van den Bergh reaction. The ultrafiltrates obtained from the centrifugation of the model sera were extracted with chloroform, and the chloroform layer was tested for bilirubin qualitatively as above. The ultrafiltrates were tested for protein qualitatively by the

[2] Armour and Company, prepared by the cold decanol fractionation procedure of Cohn. Purchased as a 30 per cent aqueous solution.

presence of a precipitate after addition of 10 per cent tricloracetic acid.

Dialysis studies. Jaundiced human sera were prepared as in the ultrafiltration studies and dialyzed in cellulose casings against running cold water, protected from light, for 5 to 72 hours. The samples were subsequently analyzed for the remaining bilirubin. These experiments were done using either the undiluted sera or samples diluted with water.

RESULTS

Spectrophotometric studies

The absorption maximum of the diluted sera was 460 mμ ± 1 mμ in 18 of the 20 patients. One of the patient's sera exhibited its absorption maxima at 465 mμ (Figure 2) and another at 470 mμ. If the human sera were diluted in 0.1 M PO_4 buffer, pH 7.4, the spectral curve of the sera was shifted slightly toward higher wave lengths in the 400 to 500 range, and the absorption peak was usually shifted from 460 mμ to 462 mμ, without change in extinction at the respective maxima.

The addition of either salicylate or sulfonamides to icteric serum samples produced alterations in their absorption spectrum. Illustrative changes are presented in Figures 1 and 2. The insert of Figure 1 illustrates the loss of optical density found in the diluted serum after each addition of sulfisoxazole and salicylate until 3 and 5 μM had been added, respectively. The spectral curves shown above the insert are the curves of the serum samples after the addition of equal volumes (0.2 ml.) of water, 400 mg. per cent sulfisoxazole and 400 mg. per cent salicylate had been made to either the control or experimental samples in the cuvettes. These volumes corresponded to the 3 μM and the 5 μM anion additions. As can be seen from these spectral curves, the anions produced a depression in the absorption at 460 mμ and a simultaneous increase in absorption over the control sample from 410 to 440 mμ with a peak at 420 mμ. The bilirubin concentration in each of the three cuvette samples, after the spectral analysis was done, gave the same values by the van den Bergh reaction.

The addition of salicylate and sulfisoxazole as illustrated in Figure 1 has now been done on sera from 20 icteric babies and similar changes have been observed in all instances. The percentage loss in absorption at the 460 mμ maximum has differed from baby to baby. The range has varied

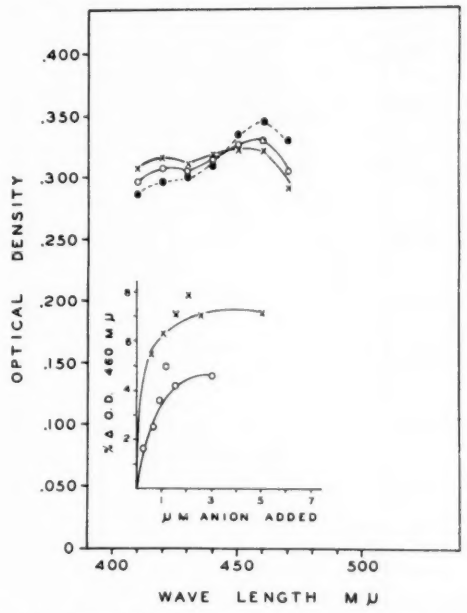


FIG. 1. SPECTRAL ABSORPTION CHANGES IN SERUM FROM A PATIENT WITH ERYTHROBLASTOSIS

Bilirubin concentration: 0.4 direct, 12 mg. per cent total. The insert is a plot of the loss in optical density at 460 mμ after each 20 λ addition of 400 mg. per cent salicylate and sulfisoxazole.

The spectral curves above represent the optical densities of the 1:20 dilution of the serum after the final water or anion additions. Total volume of addition, 0.2 ml.; bilirubin concentration in cuvettes, 0.01 μM per ml. ●---●, control sample; ○—○, sample containing 3 μM sulfisoxazole (final concentration, 1.1 μM per ml); ×—×, sample containing 5 μM sodium salicylate (final concentration, 1.8 μM per ml.).

Molar ratios of added anion to bilirubin may be estimated by multiplying μM of anion added (abscissa) by $\frac{0.4}{\text{bilirubin concentration } (\mu M \text{ per ml.})}$.

from 5 to 12 per cent with 5 μM additions of salicylate and 2 to 8 per cent for 3μM additions of sulfisoxazole. Salicylate always produced a greater loss in absorption than the sulfonamide in the sera from the 20 babies studied.

Figure 2 shows the spectral cuves of a serum in which one of three 0.2 ml. aliquots was diluted 50-fold with water alone or water and 400 mg. per cent sodium salicylate to have final salicylate concentrations of 0.4 and 40.0 mg. per cent. The depression of absorption at 465 mμ and the appearance of a shoulder at 420 to 440 mμ is apparent.

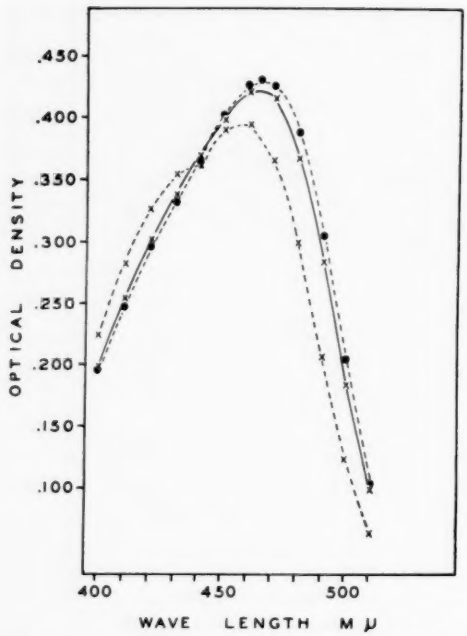


FIG. 2. SPECTRAL ABSORPTION OF SERUM FROM A TERM INFANT WITH ERYTHROBLASTOSIS

Bilirubin concentration: direct 1.9, total 22 mg. per cent. ●---●, 1:50 dilution of sera with water; ×—×, 1:50 dilution of sera with water containing sodium salicylate; final concentration, 0.025 μM per ml. Per cent Δ O.D. 465=1.9; ×---×, 1:50 dilution of sera with water containing sodium salicylate; final concentration, 2.5 μM per ml. Per cent Δ O.D. 465=10.7. Concentration of bilirubin in the diluted samples, 0.0076 μM per ml.

Also noted as a result of the salicylate was that the depression at 465 was not equaled by an increase in absorption at 420 mμ, and the whole spectral absorption was progressively shifted to lower wave lengths with higher salicylate concentrations. These two types of experiments have been done using sulfanilic acid and sodium sulfadiazine and qualitatively similar changes as described above have occurred. However much higher molar concentrations were required particularly with sulfadiazine. It is important to add that when adding sulfanilic acid to icteric sera, buffering to prevent a fall in pH was required. If the pH of the sample fell below pH 7.4, large losses in absorption all through the 400 to 500 mμ range occurred with absorption greater at 450 mμ than at 460 mμ, and the total bilirubin concentration was reduced when measured by the van den Bergh reaction. This latter change was equally well produced by simply lowering the pH of the serum sample with addition of hydrochloric acid.

Addition of heparin, sodium glucuronate or glucuronic acid to concentrations of 200 mg. per cent did not alter the spectral absorption of human sera between 400 to 500 mμ provided a fall of pH was prevented by using 0.1 M phosphate buffer as the diluent.

The absorption of the solutions in the 420 to 440 mμ ranges was unstable. If the serum samples after the anion addition were allowed to stand, the absorption at 420 to 440 became progressively diminished, and the total bilirubin was less when measured by the van den Bergh reaction.

To determine whether the spectrophotometric

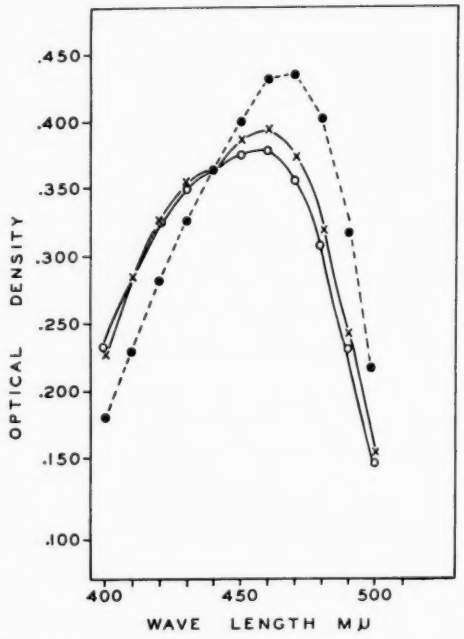


FIG. 3. SPECTRAL ABSORPTIONS OF MODEL SERUM OF BOVINE ALBUMIN BILIRUBIN SOLUTION IN PO_4 BUFFER, pH 7.4

Albumin concentration, 60 mg. per cent (0.009 μM per ml.) and bilirubin concentration, 0.55 mg. per cent (0.010 μM per ml.) were the same in each solution. ●---●, model serum alone; ○—○, model serum containing 13 mg. per cent sulfisoxazole (0.48 μM per ml.). Per cent Δ O.D. 470= 18.1. ×—×, model serum containing 13 mg. per cent salicylate (0.81 μM per ml.). Per cent Δ O.D. 470=15.4.

alteration of protein-bound bilirubin in serum caused by organic anions could be explained by changes in binding by serum albumin (or by some other protein fraction), the model serum employing bovine albumin to which bilirubin was added was studied under similar circumstances.

In Figure 3 is illustrated the absorption of model serum using bovine albumin, and it can be seen that the organic anions produced similar effects (compare Figures 2 and 3). However, sulfisoxazole had a relatively greater effect than salicylate on the model bovine serum than occurred with the human serum. Also the absorption maximum of bilirubin bound to bovine albumin was 470 mμ, not 460 mμ. It was seen in the experiments employing model sera as in human sera that although increases in concentration of organic anion produced continued loss of absorption at 460 and 470 mμ, the increases at 420 mμ were not of similar magnitudes and a shift in the whole spectral curve to shorter wave lengths occurred (see Figures 2 and 3).

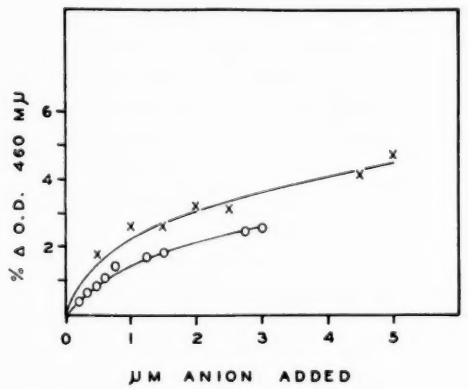


FIG. 4. SPECTRAL ABSORPTION CHANGES OF CRYSTALLINE HUMAN ALBUMIN BILIRUBIN SOLUTION IN PO_4 BUFFER, pH 7.4, AFTER ANION ADDITIONS

Albumin concentration, 120 mg. per cent (0.017 μM per ml.); bilirubin concentration, 1.0 mg. per cent (0.018 μM per ml.). ×—×, aqueous 400 mg. per cent sodium salicylate added in 20 λ (0.5 μM) amounts; ○—○, aqueous 400 mg. per cent sulfisoxazole diethanolamine added in 20 λ (0.3 μM) amounts. Each point represents the difference in optical density between a control sample and the sample to which the anion had been added.

Molar ratios of added anion to bilirubin may be estimated by multiplying μM of anion added (abscissa) by

$$\frac{0.4}{\text{bilirubin concentration } (\mu\text{M per ml.})}$$

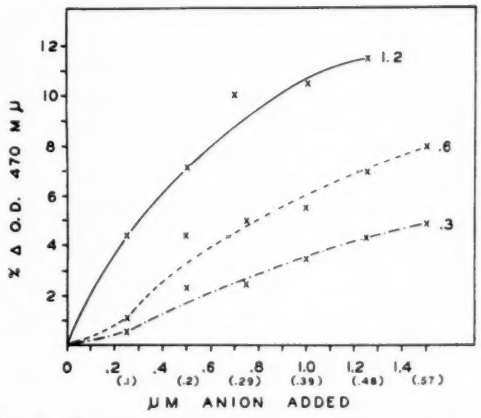


FIG. 5. BOVINE ALBUMIN BILIRUBIN MIXTURE IN PO_4 BUFFER, pH 7.4.

Each curve represents the per cent difference in optical density between a control sample and the sample to which aqueous 200 mg. per cent sodium salicylate had been added in the molar amounts indicated on the abscissa. The numbers in parentheses are the concentrations of the salicylate in μM per ml. in the cuvette samples after each addition. The numbers to the right of each curve are the molar ratio of bilirubin to albumin.

Starting concentrations: ×—×, albumin, 48 mg. per cent (0.008 μM per ml.); bilirubin, 0.52 mg. per cent (0.009 μM per ml.); ×– – –×, albumin, 120 mg. per cent (0.017 μM per ml.); bilirubin, 0.64 mg. per cent (0.010 μM per ml.); ×—·—×, albumin, 240 mg. per cent (0.035 μM per ml.); bilirubin, 0.64 mg. per cent (0.010 μM per ml.).

Molar ratios of added anion to bilirubin may be estimated by multiplying μM of anion added (abscissa) by

$$\frac{0.4}{\text{bilirubin concentration } (\mu\text{M per ml.})}$$

A single experiment done in duplicate using crystalline human albumin from Cohn fraction V[3] was also performed. Solutions of this sample of human albumin and bilirubin had an absorption maximum at 460 mμ. The extinction coefficient at 460 mμ was 4.6×10^4, similar to that at 470 mμ of comparable solutions of bovine albumin and bilirubin. The response of this solution to additions of salicylate and sulfisoxazole is illustrated in Figure 4. As with patient's sera, salicylate caused a larger depression in absorption at 460 mμ than sulfisoxazole. Considerably more organic anion was required with the crystalline human albumin-bilirubin solutions to produce significant alteration

[3] Made available through the courtesy of Dr. Morris Rosenfeld.

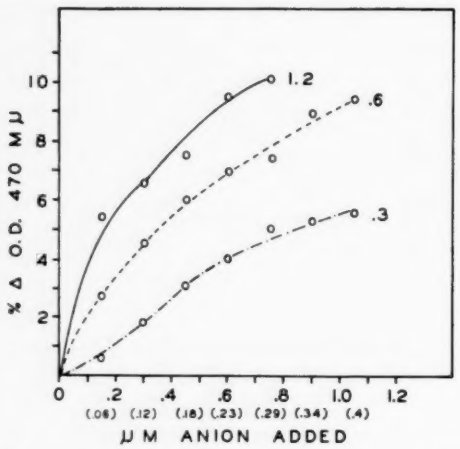


FIG. 6. BOVINE ALBUMIN BILIRUBIN MIXTURE IN PO_4 BUFFER, pH 7.4, TO WHICH AQUEOUS 200 MG. PER CENT SULFISOXAZOLE HAD BEEN ADDED

The conditions of this experiment were identical to those shown in Figure 5.

Molar ratios of added anion to bilirubin may be estimated by multiplying μM of anion added (abscissa) by $\frac{0.4}{\text{bilirubin concentration (μM per ml.)}}$.

of the absorption at 460 mμ, than was necessary with either the patient's sera (Figures 1 and 7) or the bovine-albumin-bilirubin mixtures at comparable albumin concentrations (see Figures 5 and 6.)

In order to elucidate some of the variables which influence the spectroscopic behavior of protein-bound bilirubin, the following studies were done and are illustrated in Figures 4 through 7.

A series of buffered albumin and bilirubin solutions were mixed so as to have different molar concentration ratios of bilirubin to albumin. Three 2.5 ml. samples from each molar mixture were pipetted into the cuvettes and 0.02 ml. additions of 200 mg. per cent salicylate (Figure 5) or sulfisoxazole (Figure 6) were made directly to the cuvette. The absorption at 470 mμ was measured after mixing of each addition of anion and compared to the control sample which was equally diluted with distilled water. Figures 5 and 6 show the decrease in optical density (expressed as percentage change from the control sample) plotted against the moles of added anion. The numbers in parentheses refer to the molar concentration of anion in the cuvette.

The results of these experiments indicated *a*) that a greater depression of absorption at 470 mμ per mole of anion added occurred in the solutions with higher molar ratios of bilirubin to albumin regardless of absolute bilirubin concentration; the curves of the 0.6 and 0.3 molar ratios had the same bilirubin concentration; and *b*) that a greater depression of absorption at 470 mμ was produced by increasing the anion concentration relative to that of bilirubin and albumin at all molar ratios; the change was not linear.

Figure 7 illustrates the changes in spectral absorption of serum, from a patient who had hyperbilirubinemia, upon additions of sulfisoxazole and salicylate. The molar concentration ratio of bilirubin to protein was constant, but because the serum was diluted 1 : 20 and 1 : 50 the relative concentration of anion to bilirubin and protein dif-

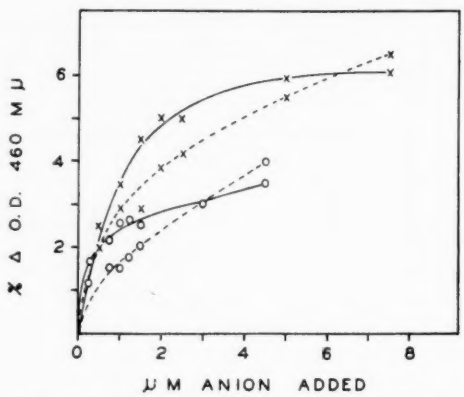


FIG. 7. THE SPECTRAL ABSORPTION CHANGES OF HUMAN SERUM AFTER ADDITION OF ORGANIC ANION

The undiluted serum had a bilirubin concentration: 0.8 direct, 15 mg. per cent total.

×—×, 1 : 50 dilution of the serum
×---×, 1 : 20 dilution of the serum
} 400 mg. per cent salicylate added in 20 λ (0.50 μM) amounts to 2.5 ml. of diluted serum.

○—○, 1 : 50 dilution of the serum
○---○, 1 : 20 dilution of the serum
} 400 mg. per cent sulfisoxazole added in 20 λ (0.30 μM) amounts to 2.5 ml. of diluted serum.

Bilirubin concentration in 1 : 20 dilution, 0.01 μM per ml.; in 1 : 50 dilution, 0.005 μM per ml.

Molar ratios of added anion to bilirubin may be estimated by multiplying μM of anion added (abscissa) by $\frac{0.4}{\text{bilirubin concentration (μM per ml.)}}$.

fered. It was seen, as with the model serum, that increasing the anion concentration of either salicylate or sulfisoxazole relative to the protein and bilirubin concentrations produced greater depressions in absorption. The importance of absolute as well as relative concentrations of anion, protein and bilirubin was illustrated by the fact that the curves for the 1 : 20 dilutions intersected those of the 1 : 50 dilutions with different slopes. Although not illustrated in Figures 5 and 6, all three curves eventually intersected one another and plateaued at about the same level.

Ultrafiltration studies

In order to ascertain that the depression in absorption at 460 mμ was due to an uncoupling of protein-bound bilirubin, the diluted human or bovine sera were ultrafiltered by centrifugation in 15 instances. The protein-free ultrafiltrates were colored yellow when salicylate, sulfadiazine, sulfisoxazole or sulfanilic acid had been added prior to ultrafiltration. Serum concentrations over 30 mg. per cent of sulfadiazine were required as compared with concentrations of 5 to 25 mg. per cent of the other anions. Spectrophotometric analysis of the filtrates showed an absorption maximum at 420 mμ. After addition of methyl alcohol and diazotized sulfanilic acid the absorption maximum at 420 was absent and a new peak at 530 to 560 mμ was present, the absorption peak of bilirubin coupled to diazotized sulfanilic acid. The shift in absorption maximum did not occur when diazotized sulfanilic acid alone was added, but the solutions became colorless, so that it was necessary to add methyl alcohol before addition of the diazo reagent. The control samples of sera ultrafiltered simultaneously had completely colorless filtrates and yielded no colored product by the van den Bergh test. Samples of sera to which glucuronic acid or sodium glucuronate had been added also had colorless filtrates when ultrafiltered and a negative van den Bergh test.

Dialysis studies

Representative results of a few dialysis experiments are shown in Table I. The sera were diluted as indicated and the anion added. An aliquot was taken and examined spectrophotometrically for the per cent Δ O.D. 460 mμ and the remainder was dialyzed.

It should be pointed out that the methodology of these two approaches are not comparable. In the spectrophotometric procedure, the uncoupled bilirubin competes with the added anion for protein binding, whereas in the dialyses the concentrations of free bilirubin and anion are lowered because they can be dialyzed from the protein phase.

The first three experiments demonstrated a greater loss in bilirubin by dialysis than the spec-

TABLE I

Effect of anions on protein-binding of bilirubin

Bilirubin concentration in original serum	Dilution of sera for dialysis	Duration of dialysis	Anion added	Concentration in diluted sera of anion after addition	Bilirubin concentration after dialysis	Per cent change from control sample	
						Of bilirubin lost by dialysis	Of optical density at 460 mμ by spectrophotometry
mg. %		*hours*		*mg. %*	*mg. %*		
1:15	0.5:0.52	72	0	0	8.2		
			Sulfisoxazole	15	6.9	−15.8	−4.3
0.6:12.5	0.5:1.5	10	0	0	12.0		
			Sulfisoxazole	3.6	11.4	− 5.0	−2.4
0.8:11	0.2:10	5	0	0	10.2		
			Sulfisoxazole	7.8	9.6	− 5.9	−1.2
			Salicylate	7.8	9.4	− 7.8	−4.7
1.2:21	0.2:10	6	0	0	17.0		
			Sulfisoxazole	0.8	17.0	0	−3.9
			Salicylate	0.8	16.0	− 5.9	−5.8

trophotometric measurement indicated. The last two experiments demonstrated that the relative effectiveness of salicylate was greater than sulfisoxazole.

After the dialyses the remaining solutions were also tested for salicylate with ferric chloride and for sulfonamide by the Bratton-Marshall technique (34). Both anions were absent at the end of the dialyses.

DISCUSSION

The spectrophotometric studies demonstrated that protein-bound bilirubin can be modified by organic anions. The new peak which appeared at 420 to 440 mμ (the absorption maximum for free bilirubin) after addition of the anion is consistent with uncoupling of bilirubin from protein. The presence of bilirubin in the ultrafiltrates of serum after the anion additions confirmed this interpretation. The reaction of the ultrafiltrates was characteristic of bilirubin rather than its glucuronide, because in aqueous acid solutions it did not couple with the diazo reagent unless alcohol was first added.

The dialysis studies also demonstrated a greater loss of bilirubin in the presence of certain organic anions. Although the bilirubin was not recovered, it was presumed to have been dialyzed from the protein phase after being uncoupled.

Some authors have found that aqueous bilirubin has an absorption peak at 440 mμ (15, 19) while others found the peak at 420 mμ (35, 36). The author has found both values. Freshly made aqueous bilirubin had an absorption maximum at 440 mμ, but on standing, even when protected from light, the peak shifted to 430 mμ and finally to 420 mμ. This shift occurred without much change in the extinction coefficient or change in bilirubin concentration by the van den Bergh reaction (37). No studies were done to investigate these differences in absorption maxima of free bilirubin, but may represent formation of molecular aggregates (38). Mesobilirubin has a spectral curve and diazo reaction very similar to bilirubin's and can be formed by reduction of bilirubin with vigorous heating over metal amalgams (39). Occurrence of such a reduction seemed unlikely.

Even though protein-bound and free bilirubin have similar molecular extinction coefficients, the uncoupling of protein-bound bilirubin as measured by loss of absorption at 460 mμ did not produce a quantitative increase at 420 to 440 mμ. Bilirubin is very insoluble and unstable in aqueous solution at pH 7.4 and may precipitate or oxidize to other derivatives when uncoupled. The progressive loss of absorption in the 420 to 440 mμ range of the serum samples containing added anion upon standing or on exposure to light was consistent with the instability of bilirubin, as was the absolute loss of bilirubin when measured by the van den Bergh reaction. Consequently, the quantitative mathematical evaluation for uncoupling as described by Klotz, Triwush and Walker (40) could not be applied. Also the amount of bilirubin uncoupled when expressed as percentage change of optical density was really a minimum value because the absorption of the free bilirubin contributed progressively more to the total absorption at 460 mμ as more anion was added. If the total spectral curves of Figure 2 could be resolved into the individual curves for free and protein-bound bilirubin, the curve of free bilirubin with 40 mg. per cent salicylate would include a greater area in the 460 mμ range than the free bilirubin curve containing 4 mg. per cent salicylate. Despite these drawbacks, the spectrophotometric studies did show qualitative similarity to the chemical laws of mass action of competition of two ions for the same locus. This was exemplified by the response of protein-bound bilirubin at different molar ratios to similar anion concentrations, and also when the bilirubin protein ratio was small, low concentrations of anion did not interfere with the protein bound bilirubin.

Both salicylate and sulfonamides have affinities for albumin through their acid radicals and compete with other organic anions for protein binding (40, 41). The major binding sites involved are the acid radicals of anions and the cationic amine groups of albumin, primarily the ε-amino groups of lysine (27). If the carboxyl groups of the proprionic acid side chains of bilirubin are concerned in the protein binding, then interference by salicylates or sulfonamides might be expected. Other groups in the bilirubin molecule may also be involved in its protein binding (42). The finding (19) that guanidination of albumin reduced its bilirubin binding capacity also indicated that more than the proprionic side chains are involved, for Klotz and Urquhart (43) found that the binding

of methyl orange was unaffected by guanidination of albumin. Because of their relatively simpler structure and greater solubility in aqueous media, one would expect that considerably greater molar concentrations of sulfonamides and salicylate relative to bilirubin would be required to interfere significantly with the protein binding of bilirubin. However, in the present studies the concentrations of salicylate and sulfisoxazole used were within the limits of blood levels seen in clinical medicine. The serum protein and bilirubin concentrations were chosen in the present studies at a level which might be present in interstitial fluid particularly of brain.

In view of the protein binding of bilirubin, it is of interest that Rosenfeld and Surgenor found that when using hematin for albumin determinations, bilirubin did not interfere (28). This suggests that if hematin is bound by the same loci as bilirubin its binding constant might be considerably greater than that of bilirubin. However, the sera Rosenfeld and Surgenor used contained mostly direct reacting bilirubin and a different type of binding has been suggested for bilirubin glucuronide (26). The author has recent spectrophotometric data showing that salicylate and sulfonamides behave toward albumin-bound hematin just as they do toward bilirubin bound to albumin, and *in vitro*, hematin and bilirubin interfere with one another for protein binding (37).

The clinical pertinence of the findings in this report is to re-emphasize that many organic ions are bound to serum albumin and may compete with one another. The data presented and the studies of Brown, Zuelzer and Robinson (44) demonstrate that the serum bilirubin concentration does not necessarily reflect the diffusible or total body bilirubin.

Although a value for a dissociation constant (or constants) of protein-bound bilirubin is not exactly known, its value is obviously small. Hence, a small absolute increase in diffusible bilirubin would represent a very large percentage change. Therefore, uncoupling of protein-bound bilirubin by organic anions would produce a large relative change in the diffusible bilirubin. This increase could shift the diffusion gradient of bilirubin and promote its diffusion out of extracellular fluids into cell cytoplasm. Administered sulfisoxazole, by its interference with protein binding of bilirubin, may prevent accumulation of bilirubin in serum thus forcing it to remain unbound or loosely bound to other tissue proteins in relatively higher concentrations in extravascular phases of body fluids. This would promote greater opportunity for filtration and diffusion through cell membranes. If the bilirubin cannot concentrate in the vascular space attached to serum proteins, the plasma bilirubin concentration will be low and will become relatively misleading in the management of hyperbilirubinemia in the neonate. The reports of kernicterus in premature infants given sulfisoxazole who had low serum bilirubins fit this concept (11, 12). Also, the reports (8, 9) of infants with high heme pigment concentrations with neurologic signs of early kernicterus and relatively low serum bilirubin levels are consistent with methemalbumin interfering with the bilirubin concentrating on serum proteins. In premature babies larger absolute amounts of bilirubin may occur in the extravascular body fluids without being reflected by high serum levels, because premature infants have lower serum albumin concentrations than full term infants (45) and, therefore, their plasma would become saturated at lower bilirubin concentrations.

The additional information of the relative saturation of serum proteins with bilirubin is also desirable. A technique such as that reported in the present studies could be applied where the amount of organic anion necessary to uncouple protein-bound bilirubin would be a measure of the relative saturation of serum proteins and, indirectly, a measure of diffusible bilirubin. This type of determination, coupled with a knowledge of the total albumin concentration, may give a more precise approach to the therapy of hyperbilirubinemia and explain some of the exceptions to the concept that prevention of kernicterus depends upon keeping the serum bilirubin below a concentration of 18 to 20 mg. per cent during the first 72 hours of life.

The fact that salicylate is rarely used in the neonate has been fortunate. Many infants have received sulfadiazine, but the concentrations, as found in the present study, which were required to interfere with protein binding of bilirubin *in vitro*, were considerably higher than those probably obtained clinically and consequently would not enhance the development of kernicterus.

SUMMARY

The protein binding of bilirubin has been studied by spectrophotometry, ultrafiltration and dialysis. It was demonstrated that the organic anions, salicylate and sulfisoxazole, could uncouple protein-bound bilirubin and thereby allow its passage through semipermeable membranes free from protein. The concentrations of anion necessary to produce this effect were within the ranges observed in clinical medicine. Sulfadiazine and sulfanilic acid were also capable of displacing protein-bound bilirubin but required higher molar concentrations. The displacement of the protein-bound bilirubin in the presence of different anion concentrations was observed to show qualitative similarities to the chemical laws of mass action of two anions competing for a common locus on the albumin molecule.

The importance of variations in protein binding and of diffusible rather than total bilirubin was emphasized in the pathogenesis of kernicterus.

REFERENCES

1. Zimmerman, H. M., and Yannet, H. Kernikterus, jaundice of the nuclear masses of the brain. Amer. J. Dis. Child. 1933, **45**, 740.
2. Mollison, P. L., and Cutbush, M. Haemolytic disease of the newborn; criteria of severity. Brit. med. J. 1949, **1**, 123.
3. Hsia, D. Y., Allen, F. H., Jr., Gellis, S. S., and Diamond, L. K. Erythroblastosis fetalis. VII. Studies of serum bilirubin in relation to kernicterus. New Engl. J. Med. 1952, **247**, 668.
4. Claireaux, A. E., Cole, P. G., and Lathe, G. H. Icterus of brain in newborn. Lancet 1953, **ii**, 1226.
5. Waters, W. J., Richert, D. A., and Rawson, H. H. Bilirubin encephalopathy. Pediatrics 1954, **13**, 319.
6. Day, R. L. Inhibition of brain respiration *in vitro* by bilirubin. Reversal of inhibition by various means. Proc. Soc. exp. Biol. (N. Y.) 1954, **85**, 261.
7. Zetterström, R., and Ernster, L. Bilirubin, an uncoupler of oxidative phosphorylation in isolated mitochondria. Nature 1956, **178**, 1335.
8. Abelson, N. M., and Boggs, T. R., Jr. Plasma pigments in erythroblastosis fetalis. I. Spectrophotometric absorption patterns. Pediatrics 1956, **17**, 452.
9. Tuttle, A. H. Serum pigment studies in newborn infants. I. Erythroblastosis fetalis. J. Dis. Child. 1955, **89**, 544.
10. Crosse, V. M., Meyer, T. C., and Gerrard, J. W. Kernicterus and prematurity. Arch. Dis. Child. 1955, **30**, 501.
11. Silverman, W. A., Andersen, D. H., Blanc, W. A., and Crozier, D. N. A difference in mortality rate and incidence of kernicterus among premature infants allotted to two prophylactic antibacterial regimens. Pediatrics 1956, **18**, 614.
12. Harris, R. C., Lucey, J. F., and MacLean, J. R. Kernicterus in premature infants associated with low concentrations of bilirubin in the plasma. Pediatrics 1958, **21**, 875.
13. Johnson, L. M. Consciousness and the chemical environment of the brain. Report of the Twenty-Fifth Ross Pediatrics Research Conference, March, 1957, 100.
14. Cohn, E. J. The chemical specificity of the interaction of diverse human plasma proteins. Blood 1948, **3**, 471.
15. Najjar, V. A., and Childs, B. Crystallization and properties of serum bilirubin. J. biol. Chem. 1953, **204**, 359.
16. Gray, C. H., and Kekwick, R. A. Bilirubin-serum protein complexes and the van den Bergh reaction. Nature 1948, **161**, 274.
17. Klatskin, G., and Bungards, L. Bilirubin-protein linkages in serum and their relationship to the van den Bergh reaction. J. clin. Invest. 1956, **35**, 537.
18. Bourrillon, R. Isolement et propriétés du complexe bilirubine-sérum albumine du sérum. Bull. Soc. Chim. biol. (Paris) 1957, **39**, 385.
19. Martin, N. H. Preparation and properties of serum and plasma proteins. XXI. Interactions with bilirubin. J. Amer. chem. Soc. 1949, **71**, 1230.
20. Pedersen, K. O., and Waldenström, J. Studien über das Bilirubin in Blut und Galle mit Hilfe von Elektrophorese und Ultrazentrifugierung. Hoppe-Seyler's Z. physiol. Chem. 1937, **245**, 152.
21. Forrai, E., and Sivo, R. Physikalisch-chemische Eigenschaften des Bilirubins in Körperflüssigkeiten. Biochem. Z. 1927, **189**, 162.
22. Gregory, R. L., and Andersch, M. The filterability of bilirubin in obstructive jaundice. J. Lab. clin. Med. 1937, **22**, 1111.
23. Snapper, I., and Bendien, W. M. On the physicochemical condition of the bilirubin in the blood serum and urine. Acta med. scand. 1938, **98**, 77.
24. Coolidge, T. B. Chemistry of the van den Bergh reaction. J. biol. Chem. 1940, **132**, 119.
25. Barac, G. Interactions of bilirubin with plasma proteins, and the state of bilirubin in normal human plasma. Arch. int. Physiol. 1953, **61**, 129.
26. Childs, B. The nature of direct bilirubin. Bull. Johns Hopk. Hosp. 1955, **97**, 333.
27. Klotz, I. M. Protein interactions *in* The Proteins, Vol. I B, H. Neurath and K. Bailey, Eds. New York, Academic Press Inc., 1953, p. 727.
28. Rosenfeld, M., and Surgenor, D. M. The hematin-binding reaction as a basis for serum albumin determination. J. biol. Chem. 1953, **199**, 911.
29. Westphal, U., and Gedigk, P. Azorubin-binding capacity of normal and pathological sera. Proc. Soc. exp. Biol. (N. Y.) 1951, **76**, 838.

30. Rutstein, D. D., Ingenito, E. F., and Reynolds, W. E. The determination of albumin in human blood plasma and serum; a method based on the interaction of albumin with an anionic dye-2-(4'-hydroxybenzeneazo) benzoic acid. J. clin. Invest. 1954, **33**, 211.
31. Blondheim, S. H. The relation between the albumin concentration of serum and its dye-binding capacity. J. Lab. clin. Med. 1955, **45**, 740.
32. Odell, G. B. *In vitro* studies of the effect of sulfonamids on bilirubin (abstract). A.M.A. Amer. J. Dis. Child. 1958, **96**, 535.
33. Malloy, H. T., and Evelyn, K. A. The determination of bilirubin with the photoelectric colorimeter. J. biol. Chem. 1937, **119**, 481.
34. Bratton, A. C., and Marshall, E. K., Jr. A new coupling component for sulfanilamide determination. J. biol. Chem. 1939, **128**, 537.
35. With, T. K. Spectral absorption of bilirubin. Measurements in pure aqueous solutions and in solutions containing human serum. Acta physiol. scand. 1945, **10**, 172.
36. Cremer, R. J., Perryman, P. W., and Richards, D. H. Influence of light on the hyperbilirubinaemia of infants. Lancet 1958, **i**, 1094.
37. Odell, G. B. Unpublished observations.
38. Jirsa, M., and Sedláček, B. Aggregation of bilirubin in some solvent-precipitant systems. Chem. Listy 1956, **50**, 520. (Cited from Chem. Abstr. 1956, **50**, 11403 h.)
39. Heilmeyer, L. Spectrophotometry in Medicine. London, A. Higler, Ltd., 1943, p. 163.
40. Klotz, I. M., Triwush, H., and Walker, F. M. The binding of organic ions by proteins. Competition phenomena and denaturation effects. J. Amer. chem. Soc. 1948, **70**, 2935.
41. Davis, B. D. The binding of sulfonamide drugs by plasma proteins. A factor in determining the distribution of drugs in the body. J. clin. Invest. 1943, **22**, 753.
42. With, T. K. The behavior of the bilirubin-albumin bond in serum on diazotization. Scand. J. clin. Lab. Invest. 1958, **10**, 188.
43. Klotz, I. M., and Urquhart, J. M. The binding of organic ions by proteins. Comparison of native and of modified proteins. J. Amer. chem. Soc. 1949, **71**, 1597.
44. Brown, A. K., Zuelzer, W. W., and Robinson, A. R. Studies in hyperbilirubinemia. 2. Clearance of bilirubin from plasma and extravascular space in newborn infants during exchange transfusion. A. M. A. Amer. J. Dis. Child. 1957, **93**, 274.
45. Darrow, D. C., and Cary, M. K. The serum albumin and globulin of newborn, premature and normal infants. J. Pediat. 1933, **3**, 573.

EFFECT OF ALTERED INTRATHORACIC PRESSURE ON RENAL HEMODYNAMICS, ELECTROLYTE EXCRETION AND WATER CLEARANCE *

By H. V. MURDAUGH, JR.,† H. O. SIEKER AND F. MANFREDI ‡

(From the Department of Medicine, Duke University School of Medicine and the Veterans Administration Hospital, Durham, N. C.)

(Submitted for publication September 15, 1958; accepted January 30, 1959)

Breathing against a continuous positive or negative pressure has been demonstrated to alter urine flow. Continuous positive pressure breathing is associated with a decrease in the rate of urine flow in dog (1) and man (2), whereas breathing against a constant negative pressure produces an increased rate of urine flow in the same species (1, 3). A decrease in urea clearance accompanies the antidiuresis of positive pressure breathing and suggests an altered rate of glomerular filtration (1). The diuresis of negative pressure breathing is not associated with marked changes in endogenous creatinine clearance or sodium and potassium excretion (1, 3).

These alterations in the rate of urine flow have been attributed to changes in distribution of blood within the chest (1). Positive pressure breathing is associated with a displacement of blood from the thorax, and breathing against a negative pressure results in an increase in the intrathoracic blood volume. Henry, Gauer and Reeves postulated that these changes in urine flow are brought about through stretch receptors in the wall of the atria and greater vessels of the thorax (4), and demonstrated that the response of the dog to negative pressure breathing could be prevented by chilling of the vagus (5). These reports suggested that such a pathway involves also a humoral substance of antidiuretic nature.

The study reported in this paper was designed to evaluate the role of renal hemodynamics and humoral factors in the production of the changes in rate of urine flow in response to pressure breathing with particular emphasis on the effects upon water clearance.

METHODS

Thirty-eight studies were performed on 12 normal volunteer medical students. Before the experiments, the subjects were trained to breathe comfortably against a continuous pressure and to void freely, emptying the bladder at 15 minute intervals. Proposed subjects who did not so adapt themselves were not used for the studies.

The rate of urine flow was observed for two to four hours under the circumstances of the particular experiment to insure a stable control rate. Fifteen minute urine collections were then obtained throughout three to six control periods, during 30 minutes of pressure breathing, and afterwards until the rate of urine flow returned to the control value.

Pressure breathing consisted of a continuous positive pressure in the range of 24 to 26 mm. Hg or continuous negative pressure in the range of 18 to 22 mm. Hg. Continuous positive pressure was administered through a positive pressure demand valve system. Continuous negative pressure was obtained by breathing room air through a system which incorporated a large drum in which the negative pressure was maintained by a suction pump (3). The positive pressure breathing experiments were carried out with the subject in the seated position throughout the study (control, test, and recovery periods). The negative pressure breathing experiments were performed with the subject in the horizontal position during the entire study. Continuous positive pressure was administered during the states of water diuresis in nine subjects, alcohol diuresis in nine subjects, and mannitol osmotic diuresis in five subjects. Alcohol diuresis was accomplished by instituting a water diuresis and then giving 20 ml. absolute alcohol by mouth and adding 10 ml. absolute alcohol to the inulin infusion 30 to 45 minutes before the positive pressure breathing was started. Osmotic diuresis was obtained by administration of mannitol in the priming infusion and by a 15 per cent mannitol maintenance infusion. Negative pressure was used during the postabsorptive state in nine subjects who had received only one glass of water every two hours for the six hours prior to the study, and following vasopressin

* Supported in part from the Veterans Administration Clinical Investigator Program, in part from a United States Public Health Service Grant (A-1596) and in part from Life Insurance Medical Research Fund.

† Done during the tenure as a VA Clinical Investigator. Present address: Department of Medicine, University of Alabama Medical Center and Veterans Administration Hospital, Birmingham, Ala.

‡ Research Fellow of the American Heart Association, 1958–59.

(Pitressin®) or hypertonic salt administration in five subjects. Subjects were studied in both the morning and evening, thus avoiding the problem of diurnal variation.

Standard renal clearance techniques were used for the measurement of inulin, sodium para-aminohippurate (PAH), sodium, potassium, osmolar and free water clearances. Prime and maintenance infusions of inulin and PAH were administered at a constant rate with an infusion pump and the administered load was calculated to obtain plasma concentrations of about 20 mg. per cent inulin and 1 to 2 mg. per cent PAH. Blood samples were obtained in heparinized syringes via an indwelling needle for the determination of plasma osmolality and plasma inulin, PAH, sodium and potassium concentrations. In some subjects urea clearances were also performed to evaluate further the content of the urine solute. Inulin clearance was used as a measure of glomerular filtration rate (GFR). PAH clearance was divided by 0.9 to obtain renal plasma flow (RPF). Free water clearance was calculated from the osmolar clearance and the minute urine volume.

A plasma filtrate was made by the cadmium sulfate method of Fujita and Iwatake (6). Inulin was determined by the resorcinol method as modified by Schreiner (7). PAH determinations were carried out using the method of Selkurt (8) with the N-napthyl ethylenediamine dihydrochloride recrystallized by hydrochloric acid as described by Bratton and Marshall (9). Osmolality measurements were performed using a Fiske Osmometer Model B. Sodium and potassium determinations were carried out using the internal standard flame photometer. Samples were analyzed for urea by the micro-diffusion method of Conway and O'Malley (10).

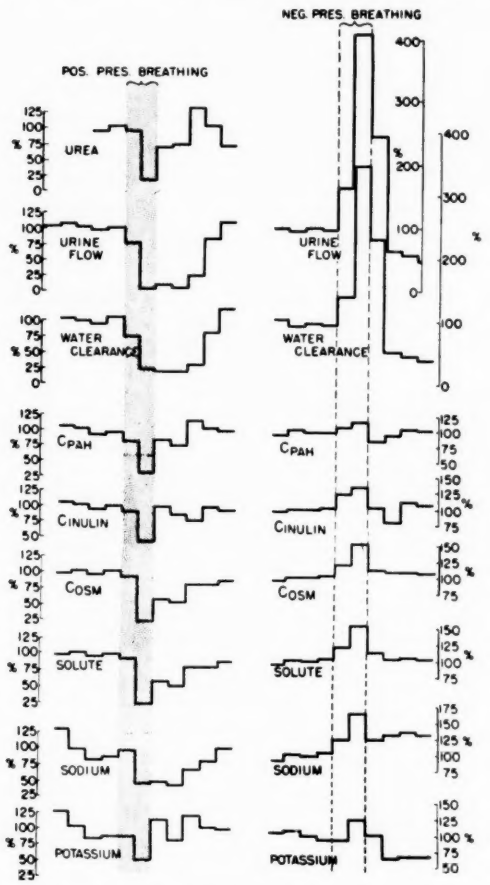


FIG. 1. GRAPHIC PRESENTATION OF DATA ON A SINGLE SUBJECT UNDERGOING CONTINUOUS POSITIVE AND CONTINUOUS NEGATIVE PRESSURE BREATHING

Percentile values are used to compare relative changes. For quantitative values refer to Subject D.H. in Tables I and III.

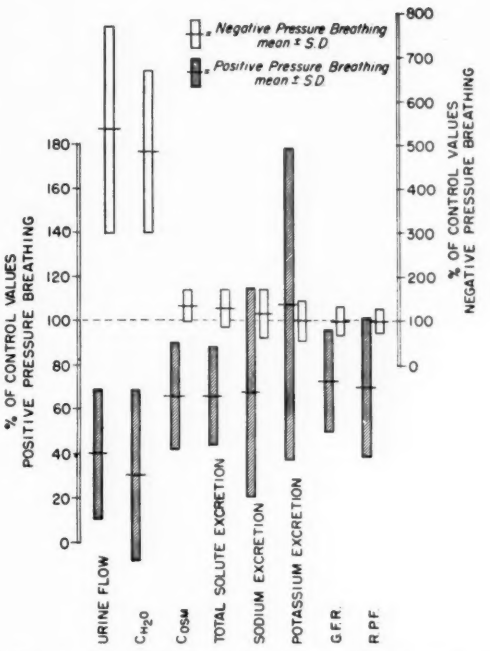


FIG. 2. SUMMARY DIAGRAM OF ALL STUDIES TABULATED IN TABLES I AND III, GIVING CONCISE COMPARISON OF THE EFFECTS OF CONTINUOUS POSITIVE AND CONTINUOUS NEGATIVE PRESSURE BREATHING

To obtain the percentile change for free water clearance (C_{H_2O}) during negative pressure breathing, the change in free water clearance was compared to the control rate of urine flow.

RESULTS

An example of the results obtained during both positive and negative pressure breathing experi-

TABLE I

Positive pressure breathing experiments *

Subject	Urine flow		Free water clearance†		Osmolal clearance		Total solute		Sodium		Potassium		GFR		RPF		Urea‡	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
	ml./min.		*ml./min.*		*ml./min.*		*mOsm./min.*		*mEq./min.*		*mEq./min.*		*ml./min.*		*ml./min.*		*mg./min.*	
D. H.	15.3	0.4	11.2	−1.3	4.28	2.23	1.21	0.61	0.137	0.064	0.030	0.024	103	45	633	200		
P. H.	15.0	2.3	11.2	−1.2	3.80	3.72	1.06	0.96	0.150	0.215	0.073	0.142						
S. A.	11.6	7.8	7.8	3.4	3.05	2.94	0.86	0.73	0.154	0.121	0.026	0.032	124	106	650	529	19.6	15.2
R. A.	15.0	7.0	10.7	5.5	3.20	1.77	0.86	0.49	0.094	0.056	0.034	0.040	129	84	835	446	28.0	15.0
T. K.	16.2	11.7	12.4	9.0	3.78	2.68	0.64	0.68	0.124	0.084	0.033	0.033	156	119	1,023	858		
B. J.	14.4	1.1	10.4	−0.3	3.93	1.43	1.10	0.40										
D. G.	11.2	7.2	8.1	5.0	3.02	2.19	0.84	0.62	0.104	0.071	0.048	0.071	134	120	529	482	18.0	14.8
P. Ho.	15.8	10.0	12.6	7.6	3.27	2.42	0.90	0.67	0.085	0.040	0.051	0.083	140	120	802	711		
D. W.	10.3	2.5	8.0	1.6	2.30	0.92	0.64	0.26	0.157	0.032	0.021	0.020	122	68	609	307	13.4	6.1
Mean	13.9	5.6	10.3	3.2	3.40	2.26	0.90	0.60	0.126	0.085	0.040	0.056	130	95	726	504	19.8	12.8
S. D.	2.2	4.1	1.9	3.9	0.60	0.83	0.20	0.20	0.028	0.059	0.017	0.041	17	30	170	226		
p value	<0.001		<0.001		<0.01		<0.01		>0.05		>0.2		<0.01		<0.01			
Same data expressed per 100 ml. GFR																		
Mean	10.6	6.3	8.1	3.8	2.57	2.47	0.68	0.67	0.098	0.076	0.026	0.045			556	519		
S. D.	2.2	3.1	1.6	3.9	0.73	1.18	0.24	0.32	0.029	0.038	0.008	0.016			91	109		
p value	<0.01		<0.01		>0.5		>0.5		>0.1		<0.01				>0.4			

* The controls represent the average of the three or four urine collections immediately before the institution of pressure breathing. The "test" collection represents that urine sample being collected at the termination of the 30 minutes of pressure breathing.

† Free water clearance was calculated using the equation, C_{H_2O} (ml. per minute) equals urine (ml. per minute) minus C_{osm} (ml. per minute). Thus, negative values represent $T^c_{H_2O}$.

‡ Urea data for Subject D. H. is presented in Figure 1, but since urea determinations were done on only two controls the data were not included in this table.

ments in the same subject is depicted in Figure 1. This subject had the most pronounced changes in GFR and RPF encountered during positive pressure breathing. In general, electrolyte excretion and total solute clearance paralleled the changes in the GFR. The changes in free water clearance, however, with resultant changes in the rate of urine flow were more pronounced and of longer duration than the changes in GFR.

Positive pressure breathing

Continuous positive pressure was administered to nine subjects during water diuresis with the demonstration of a prompt antidiuresis (Table I). The data for these nine subjects are presented in the summary diagram, Figure 2. For the group it can again be seen that solute excretion compares well with glomerular filtration rate. The most pronounced change occurred in the free water clearance with a resultant decrease in the rate of urine flow. This represented a true antidiuresis and was accompanied by increase in osmolal urine to plasma (U/P) ratio.

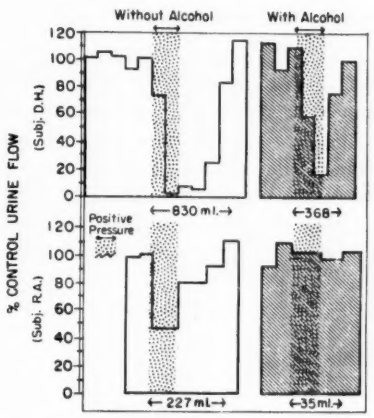


FIG. 4. TWO EXAMPLES OF THE EFFECT OF ALCOHOL ADMINISTRATION ON THE ANTIDIURESIS OF POSITIVE PRESSURE BREATHING TO SHOW DECREASE IN DEGREE OR DURATION OF ANTIDIURESIS

The values between the arrows represent the difference between observed urine flow during the period depicted by the arrows and the urine flow that would have occurred during the same period if urine flow had remained at control rate.

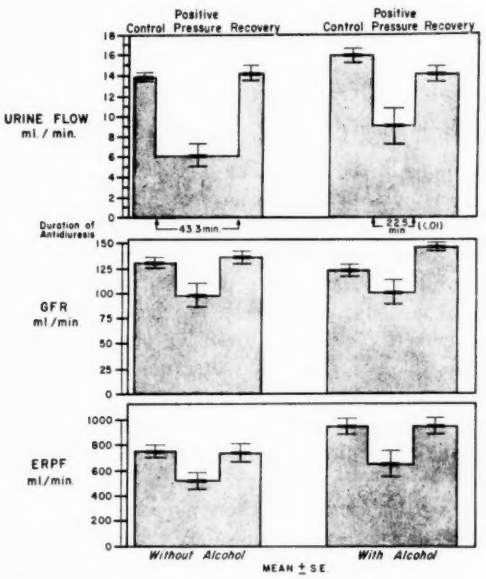


FIG. 3. SUMMARY DIAGRAM OF NINE SUBJECTS COMPARING RESPONSE TO 30 MINUTES OF CONTINUOUS POSITIVE PRESSURE BREATHING WITH AND WITHOUT ALCOHOL

The duration and degree of change in the rate of urine flow are depicted. The p value for the significance of the change in duration is noted.

As shown in Table I, there was a statistically significant decrease in the rate of urine flow, free water and osmolar clearance, sodium excretion, GFR and RPF. When the data were recalculated and expressed per 100 ml. GFR, only the rate of urine flow and the free water clearance were significantly decreased relative to GFR. The potassium excreted per 100 ml. GFR was significantly increased.

These subjects were studied again during alcohol diuresis and during mannitol osmotic diuresis. The administration of alcohol during water diuresis caused partial or complete inhibition of the antidiuretic effect of positive pressure breathing even though the change in GFR was similar to the group with water diuresis depicted in Table I (Figure 3). Figure 4 presents two examples of the inhibition by alcohol.

During osmotic diuresis with rates of urine flow as high as 16 ml. per minute there was no antidiuresis during or following positive pressure breathing, although there were decreases in GFR (Table II).

Negative pressure breathing

Eight of nine normally hydrated subjects had a diuresis with dilution of the urine in response to continuous negative pressure breathing (Table III). The data for the eight subjects who had demonstrable diuresis are presented in the summary diagram, Figure 2. Although there were slight increases in solute excretion, it is apparent that the most pronounced change that occurred was an increase in free water clearance which was accompanied by a decrease in osmolal U/P ratios to less than one.

TABLE II

Positive pressure breathing during osmotic diuresis *

Subject	Urine flow		GFR	
	Control	Test	Control	Test
	ml./min.		*ml./min.*	
D. H.	3.60	3.93		
B. K.	3.42	3.13	94	58
V. M.	15.14	15.10		
P. H.	5.30	5.00		
B. J.	5.88	4.30	112	82

* Control values represent the average of three to six urine collections obtained prior to institution of positive pressure breathing. The "test" collection represents the urine being collected at the termination of 30 minutes of pressure breathing.

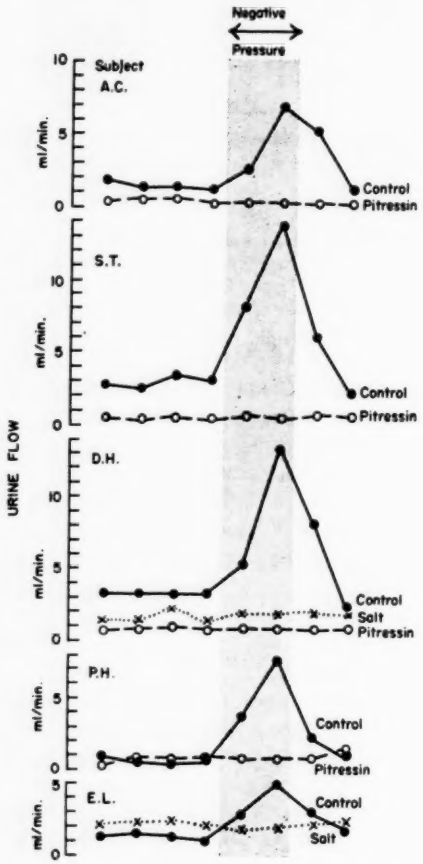


FIG. 5. THE PLOT OF 15 MINUTE URINE COLLECTIONS TO SHOW THE EFFECT OF NEGATIVE PRESSURE BREATHING ON URINE FLOW

The solid dots (●) represent the subject's normal response to negative pressure breathing, the open circles (○) represent response when given vasopressin before the institution of negative pressure breathing, and the (×)'s represent the response to negative pressure breathing after infusion of hypertonic salt solution.

The administration of vasopressin was found to prevent the diuresis in response to negative pressure breathing (Figure 5). Two subjects were given a continuous intravenous infusion of 2.5 per cent salt for three hours at 2 ml. per minute. There was noted an increase in plasma osmolality of 3 per cent, and negative pressure breathing did not alter the rate of urine flow (Figure 5).

DISCUSSION

The results of this study confirm earlier demonstrations of the alterations in urine flow that are associated with changes in intrathoracic pressure (1–3, 11–13). Although earlier reports suggested that water excretion was altered independent of electrolyte excretion and unrelated to changes in renal hemodynamics, these two factors were not completely evaluated. From the present study it is possible to conclude that alterations in electrolyte excretion and renal hemodynamics did occur during positive pressure breathing but are not of the magnitude or always in the direction of the changes in water clearance and rate of urine flow. On the other hand, electrolyte excretion and renal hemodynamics were not significantly changed with the diuresis of negative pressure breathing.

By controlling or taking into account such variables as fluid and solute intake, position, comfort and diurnal rhythm, there remained two factors to be considered in the production of an altered rate of urine flow. These were changes in antidiuretic

TABLE III

Negative pressure breathing experiments *

Subject	Urine flow		Free water clearance		Osmolal clearance		Total solute		Sodium		Potassium		GFR		RPF		Urea	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
	ml./min.		*ml./min.*		*ml./min.*		*mOsm./min.*		*mEq./min.*		*mEq./min.*		*ml./min.*		*ml./min.*		*mg./min.*	
D. H.	3.3	13.3	0.04	8.2	3.28	5.07	0.94	1.46	0.256	0.423	0.038	0.048	97	132	724	842		
P. H.	0.5	7.9	−1.2	5.2	1.69	2.68	0.46	0.71	0.081	0.091	0.036	0.077	141	113	961	962		
S. T.	3.0	13.6	−1.0	9.0	3.95	4.64	1.15	1.35	0.323	0.326	0.105	0.064	112	136	700	674		
A. C.	1.4	6.9	−1.3	3.8	2.74	3.13	0.77	0.88	0.207	0.193	0.038	0.027	106	66	586	610	15.4	16.6
H. S.	0.7	2.8	−0.8	0.8	1.45	1.99	0.43	0.58	0.103	0.136	0.019	0.018	96	108	394	465	7.8	11.5
D. Hi.	1.6	4.9	−1.4	1.6	2.95	3.26	0.84	0.93	0.246	0.258	0.043	0.045	110	97	543	604	16.7	17.0
J. W.	1.5	8.6	−0.7	5.6	2.24	2.98	0.78	0.84			0.034	0.026	179	205	728	846	10.5	12.8
D. W.	0.8	10.0	−1.6	6.8	2.40	3.20	0.66	0.88	0.178	0.221	0.037	0.037	142	150	779	703	8.9	10.8
Mean	1.6	8.5	−1.0	5.1	2.54	3.37	0.75	0.95	0.199	0.235	0.043	0.043	123	126	677	713	11.9	13.5
S. D.	1.0	3.7	0.5	2.9	0.82	1.01	0.24	0.31	0.087	0.113	0.026	0.020	28	41	170	161		
p value	<0.001		<0.001		<0.01		<0.02>0.01		>0.1		>0.5		>0.5		<0.05>0.02			
Same data expressed per 100 ml. GFR																		
Mean	1.4	7.0	−0.8	4.1	2.23	2.89	0.65	0.82	0.180	0.210	0.037	0.037			558	605		
S. D.	1.1	2.9	0.4	2.1	0.94	1.12	0.26	0.32	0.086	0.092	0.022	0.027			122	193		
p value	<0.001		<0.001		<0.05>0.02		<0.05>0.02		>0.2		>0.5				>0.4			

* The controls represent the average of the three or four urine collections immediately before the institution of pressure breathing. The "test" collection represents the peak urine flow and was either the urine collection being obtained at the termination of the pressure breathing or the first urine collection obtained after pressure breathing was completed.

hormone activity with resultant changes in water clearance or alterations in renal hemodynamics.

With positive pressure breathing a decrease in the GFR and RPF occurred. This decrease was less in degree and duration than the change in water clearance and rate of urine flow, and it occurred during osmotic diuresis when no antidiuresis was noted in response to positive pressure breathing.

Alcohol is reported to inhibit the release of antidiuretic hormone (ADH) from the posterior pituitary and thus block an antidiuretic response dependent upon ADH release (14). During alcohol-water diuresis partial or complete inhibition of the response to positive pressure breathing occurred even though the GFR was decreased. Van Dyke (15) has demonstrated that the human posterior pituitary contains about 15,000 mU of ADH, and that only 0.5 to 2.0 mU is required to cause antidiuresis. This may be the explanation for the failure of alcohol to prevent completely the antidiuretic response to positive pressure breathing.

Since mannitol-induced osmotic diuresis is accompanied by pronounced ADH activity, it was felt that an added stimulus for the release of ADH would not cause a decrease in the rate of urine flow during prominent osmotic diuresis. It was realized that a decrease in GFR, by decreasing the solute load in the glomerular filtrate, might be associated with a decrease in the degree of osmotic diuresis. As it evolved, however, there did occur a decrease in GFR, but a decrease in the rate of urine flow did not occur.

When free voiding is used for urine collections in clearance procedures, the accuracy of the clearance for each individual urine collection period is less than that obtained if an indwelling catheter and terminal washouts are used. This error is, of course, accentuated with decreasing rate of urine flow. As a consequence, the decrease in GFR and other clearances during antidiuresis may have appeared more exaggerated than the changes which occurred. For this reason the data were recalculated and expressed per 100 ml. GFR, thus cancelling out the error of time and volume when comparing changes that occurred in the different clearance measurements. By this means it was apparent that the only significant decreases relative to GFR that occurred during positive pressure breathing were the rate of urine flow and the free water clearance. The presence of such differential changes and the increase in urine concentration demonstrate that the changes found were not a phenomenon of less accurate urine collections. On the other hand, the decreases in GFR which occurred during alcohol and osmotic diuresis when the rate of urine flow did not decrease demonstrates that a decrease in GFR in response to positive pressure breathing is real and not entirely a function of less complete urine collections.

From these considerations it is believed that the antidiuresis of positive pressure breathing is primarily related to ADH activity although a decrease in GFR may play a role.

It is of interest that the potassium excretion, expressed per 100 ml. GFR, increased during positive pressure breathing even though an antidiuresis occurred. The explanation for the increase in the potassium excretion is not clear.

It is apparent from the summary diagram of the data (Figure 2) that the diuresis of negative pressure breathing is not due to change in GFR or RPF, but is due to an increase in the free water clearance with resultant increase in the rate of urine flow. Moreover, administration of vasopressin or an increase in plasma osmolality prior to negative pressure breathing inhibited the diuretic response. Therefore, it is concluded that a mechanism producing these effects must involve alteration of antidiuretic hormone activity.

Some of the effects of pressure breathing on the circulation have been studied but most of the investigation has been confined to anesthetized animals. In general, positive pressure breathing has been reported to decrease the central blood volume, cardiac output and arterial pressure. Negative pressure breathing should engorge the central blood reservoir, but reports on the effects on cardiac output and blood pressure have been conflicting. Recently, studies from this laboratory in conscious adults have demonstrated that positive pressure breathing decreases the central blood volume and cardiac output and that negative pressure breathing has the reverse effect (16). Neither positive nor negative pressure breathing altered plasma volume during the first half hour. It was concluded that within certain limits intrathoracic pressure and cyclic variation in that pressure are important in the distribution of blood volume. Pressure breathing, therefore, lends itself

to experiments designed to investigate volume receptor areas.

The relationship between altered blood volume and changes in renal excretion of water and electrolytes have been recognized (17). However, there has been controversy regarding the site of a receptor area which can detect alterations in blood volume and initiate changes in rate of urine flow. Harrison and co-workers have reported a natriuresis and a moderate increase in urine flow with neck cuff inflation (18, 19). They have postulated that a volume receptor area in the brain is responsible for initiating these changes. Other investigators have been unable to confirm these experiments (20–23). Epstein, Post and McDowell (24) demonstrated a natriuresis with occlusion of A-V fistulae and postulated that the volume receptor was in the arterial side of the vascular tree. Harrison and Epstein were both reporting manipulations that produced primarily a natriuresis. The response to pressure breathing, however, consists primarily of alterations in free water clearance and does not appear to be the same phenomenon.

It has been suggested in earlier reports that the intrathoracic portion of the cardiovascular system contains a volume receptor area (1, 3). Further study has demonstrated that the left atrium is one such receptor area (4, 5). The present observations suggest that volume receptor areas in the thorax are responsible primarily for altering water clearance by the kidney. Hyperventilation (21) and pulsatile stimuli to the lungs (25) have also been associated with a water diuresis.

SUMMARY

The effect of continuous pressure breathing upon renal function and water clearance has been studied. It was found that positive pressure breathing produces a decrease in urine flow resulting primarily from a decrease in free water clearance, with decrease in glomerular filtration rate (GFR) as a contributing factor. Negative pressure breathing is associated with an increase in free water clearance with resultant increase in the rate of urine flow without changes in GFR It is concluded that altered antidiuretic hormone (ADH) activity is the primary mechanism by which continuous pressure breathing changes the rate of urine flow.

ADDENDUM

Since this manuscript was submitted, the following reference has appeared in print: Boyland, J. W., and Antkowiak, D. E. Mechanism of diuresis during negative pressure breathing. J. appl. Physiol. 1959, **14**, 116.

REFERENCES

1. Gauer, O. H., Henry, J. P., Sieker, H. O., and Wendt, W. E. The effect of negative pressure breathing on urine flow. J. clin. Invest. 1954, **33**, 287.
2. Drury, D. R., Henry, J. P., and Goodman, J. The effects of continuous pressure breathing on kidney function. J. clin. Invest. 1947, **26**, 945.
3. Sieker, H. O., Gauer, O. H., and Henry, J. P. The effect of continuous negative pressure breathing on water and electrolyte excretion by the human kidney. J. clin. Invest. 1954, **33**, 572.
4. Henry, J. P., Gauer, O. H., and Reeves, J. L. Evidence of the atrial location of receptors influencing urine flow. Circulat. Res. 1956, **4**, 85.
5. Henry, J. P., and Pearce, J. W. The possible role of cardiac atrial stretch receptors in the induction of changes in urine flow. J. Physiol. (Lond.) 1956, **131**, 572.
6. Fujita, A., and Iwatake, D. Bestimmung des echten Blutzuckers ohne Hefe. Biochem. Z. 1931, **242**, 43.
7. Schreiner, G. E. Determination of inulin by means of resorcinol. Proc. Soc. exp. Biol. (N. Y.) 1950, **74**, 117.
8. Selkurt, E. E. Measurement of renal blood flow. Meth. med. Res. 1949, **1**, 196.
9. Bratton, A. C., and Marshall, E. K., Jr. A new coupling agent for sulfanilamide determination. J. biol. Chem. 1939, **128**, 537.
10. Conway, E. J., and O'Malley, E. Microdiffusion methods. Ammonia and urea using buffered absorbents. Biochem. J. 1942, **36**, 655.
11. Silvette, H. Some effects of low barometric pressures on kidney function in the white rat. Amer. J. Physiol. 1943, **140**, 374.
12. Surtshin, A., Hoeltzenbein, J., and White, H. L. Some effects of negative pressure breathing on urine excretion. Amer. J. Physiol. 1955, **180**, 612.
13. Knoefel, P. K., Handley, C. A., and Huggins, R. A. Renal functions during positive pressure respiration. Proc. Soc. exp. Biol. (N. Y.) 1953, **82**, 430.
14. Eggleton, M. G. The diuretic action of alcohol in man. J. Physiol. (Lond.) 1942, **101**, 172.
15. Van Dyke, H. B. The regulation of water excretion by the neurohypophysis. Bull. N. Y. Acad. Med. 1953, **29**, 24.
16. Kilburn, K. H., Sieker, H. O., and Murdaugh, H. V., Jr. Hemodynamic effects of pressure breathing (abstract). Clin. Res. 1958, **6**, 313.
17. Zuidema, G. D., Clarke, N. P., Reeves, J. L., Gauer, O. H., and Henry, J. P. Influence of moderate changes in blood volume on urine flow. Amer. J. Physiol. 1956, **186**, 89.

18. Lewis, J. M., Jr., Buie, R. M., Sevier, S. M., and Harrison, T. R. The effect of posture and of congestion of the head on sodium excretion in normal subjects. Circulation 1950, **2**, 822.
19. Viar, W. N., Oliver, B. B., Eisenberg, S., Lombardo, T. A., Willis, K., and Harrison, T. R. The effect of posture and of compression of the neck on excretion of electrolytes and glomerular filtration: Further studies. Circulation 1951, **3**, 105.
20. Fishman, R. A. The failure of intracranial pressure-volume change to influence renal function. J. clin. Invest. 1953, **32**, 847.
21. Barbour, A., Bull, G. M., Evans, B. M., Jones, N. C. H., and Logothetopoulous, J. The effect of breathing 5 to 7 percent carbon dioxide on urine flow and mineral excretion. Clin. Sci. 1953, **12**, 1.
22. Netravisesh, V. Effects of posture and of neck compression on outputs of water, sodium and creatinine. J. appl. Physiol. 1953, **5**, 544.
23. Kelser, G. A., Izlar, H. L., Estes, E. H., and Warren, J. V. A comparison of the effects of posture and neck compression on the excretion of sodium. Clin. Res. Proc. 1955, **3**, 136.
24. Epstein, F. H., Post, R. S., and McDowell, M. The effect of an arteriovenous fistula on renal hemodynamics and electrolyte excretion. J. clin. Invest. 1953, **32**, 233.
25. Love, A. H. G., Roddie, R. A., Rosensweig, J., and Shanks, R. G. The effect of pressure changes in the respired air on the renal excretion of water and electrolytes. Clin. Sci. 1957, **16**, 281.

THE EFFECTS OF ADRENAL STEROIDS AND POTASSIUM DEPLETION ON THE ELABORATION OF AN OSMOTICALLY CONCENTRATED URINE *

By GERHARD GIEBISCH † AND RODOLFO LOZANO ‡ WITH THE TECHNICAL ASSISTANCE OF ANITA MACEO

(From the Department of Physiology, Cornell University Medical College, New York, N. Y.)

(Submitted for publication August 19, 1958; accepted January 29, 1959)

The investigation reported in this paper is concerned with the effects of desoxycorticosterone acetate (DCA), hydrocortisone and dietary potassium depletion on the elaboration of an osmotically concentrated urine in the hydropenic state. Loeb and his associates (1, 2) demonstrated that dogs maintained on large amounts of DCA develop a diabetes insipidus-like picture. Since then, renal function studies in man (3–5), dogs (6) and rats (7, 8) have shown that the ability of the kidney to concentrate the urine is markedly reduced in states of adrenal hyperfunction and potassium deficiency. The mechanism by which polyuria is produced under these conditions has not been established. In particular, the role of potassium deficiency is still a matter of controversy. Thus, contrary to Loeb and co-workers (1, 2), who were unable to prevent the development of polyuria in their DCA-treated animals by feeding potassium chloride, some authors maintain (9) that the increased loss of potassium following the administration of adrenal steroids is responsible for the impairment of water conservation. Also, there is no agreement on the sensitivity of the kidney to vasopressin (Pitressin®) during various states of potassium depletion (7, 9).

The present study is an attempt to clarify some of these points. We have, in particular, studied the formation of a concentrated urine during osmotic diuresis in the hydropenic state. According to current concepts, an osmotically concentrated urine can be visualized as being made up of an isosmotic portion less the amount of water abstracted from it to produce hypertonicity (10, 11). This latter moiety has been designated $T^c_{H_2O}$ (10, 11) or water economy (12) and direct evidence indicates that the collecting duct system is the site where the process of final water abstraction occurs (13, 14). Contrary to findings obtained on control animals in which variations of $T^c_{H_2O}$ are fairly small over a wide range of urine flows, our results show that DCA administration produces a significant and progressive reduction of that amount of water as urine flow increases. Also, at higher flow rates the occurrence of marked hypotonicity was a frequent finding, thus indicating that the fluid reaching the site of final water abstraction was not rendered isosmotic during its passage along the distal tubule and collecting duct (13, 14).

METHODS

Thirty-eight experiments were performed on nine female mongrel dogs ranging in weight from 17 to 25 Kg.[1] Anesthesia was induced and maintained by appropriate amounts of sodium pentobarbital given intravenously. In all animals data on osmolality of urine and plasma at low urine flow rates and during osmotic diuresis were obtained before and after treatment with various hormones and diets. Thus, each animal served as its own control. Since, in particular, variations in the protein intake have been shown to influence the concentrating ability of the kidney (15), a diet of constant composition was supplied to all animals during each sequence of experiments. Usually this diet was a mixture of horse meat and meal having a similar composition to that used in a previous investigation (16). Additional sodium chloride in amounts varying between 86 and 172 mEq. per day was given during treatment with DCA and hydrocortisone (16–18). Dietary potassium depletion was induced by feeding a synthetic diet having the same composition as that used in a previous investigation (16).

Desoxycorticosterone acetate (DCA) in sesame oil (Cortate®, Schering) was given intramuscularly in a dose of 1.0 mg. per Kg. per day for periods lasting 6 to

* Aided by a grant from the National Heart Institute, Bethesda, Md.

† This work was done during the tenure of an Established Investigatorship of the American Heart Association.

‡ Fellow of the Rockefeller Foundation. Present address: Instituto de Biologia Andina, Apartado 1116, Lima, Peru.

[1] We are indebted to Mr. L. Glasser and Mr. F. J. Erdman, Jr., who carried out eight experiments under our supervision in the course of second year project teaching in physiology.

TABLE I

Results of a typical experiment showing effect of mannitol diuresis on elaboration of a concentrated urine in the hydropenic state (Experiment 12, 23 Kg. dog) *

Time	Urine flow	Urine	Plasma	Osmotic U/P ratio	C_{osm}	$T^c_{H_2O}$	GFR
min.	*ml./min.*	*mOsm./ Kg. H_2O*	*mOsm./L.*		*ml./min.*	*ml./min.*	*ml./min.*
0	No infusion						
0– 30	0.19	2,100	302	6.96	1.34	1.15	
30– 50	0.20	2,133	303	7.03	1.41	1.21	
55	Prime: Creatinine, 1.5 Gm.; vasopressin, 50 mU; H_2O, 20 ml.						
60	Sustaining I: Infuse at 0.4 ml./min. creatinine, 7.0 Gm.; vasopressin, 400 mU; H_2O, 120 ml.						
62	Sustaining II: Infuse at increasing rates 100 Gm. mannitol; 4.5 Gm. NaCl; 500 ml. H_2O.						
50– 90	1.41	1,059	305	3.47	4.90	3.49	82.1
90–100	2.12	960	309	3.10	6.58	4.46	80.2
100–110	3.32	703	311	2.26	7.50	4.18	79.3
110–120	4.16	610	315	1.94	8.08	3.92	80.4
120–130	6.50	486	330	1.47	9.55	3.05	74.8
130–140	7.70	456	335	1.36	10.48	2.78	76.2
140–150	8.52	436	340	1.28	10.90	2.38	79.1
150–160	9.00	423	342	1.24	11.13	2.13	78.8

* Preinfusion plasma potassium level: 3.80 mEq. per L.

24 days. Hydrocortisone (Cortef®, Schering) was administered intramuscularly in doses of 7.5 and 10 mg. per Kg. per day for periods of 6 to 13 days. On the day of the experiment, 100 to 150 mg. were included in the priming and sustaining infusion.

Prior to all experiments the animals were rendered hydropenic by deprivation of water for 48 hours and withholding of food for 24 hours. After such preparation minimal rates of urine flow were observed, and one to three urine samples were collected over a period of from 30 to 90 minutes. Subsequently, osmotic diuresis was induced by intravenous administration of a 20 per cent mannitol solution given at a rate varying between 1 and 9 ml. per minute. This infusion contained a small amount of sodium chloride (4.5 Gm. per L.) to avoid marked changes in the plasma sodium concentration (19). Simultaneously, a second infusion was given at a rate of 0.4 ml. per minute to maintain the plasma level of creatinine and vasopressin (Pitressin®, Parke, Davis and Co.) at a constant level. An appropriate priming infusion containing creatinine and vasopressin (40 to 340 mU) preceded this second infusion. An equilibration period of 20 to 65 minutes was allowed after the start of these two separate sustaining infusions before urine samples were collected. Almost all observations during mannitol infusions were made during increasing osmotic diuresis. Urine was collected by means of an indwelling catheter, residual urine being expelled by air. No washout fluid was used. Blood samples were drawn into heparinized syringes at the midpoint of each collection period either from the femoral artery or the jugular vein.

Plasma and urine samples were analyzed for creatinine (20), the clearance of which was used as a measure of glomerular filtration rate. Urine and plasma osmolality were determined cryoscopically using a Fiske osmometer. Analyses for potassium were done on preinfusion plasma samples by methods previously described (16). Glucose T_m and glucose titrations were performed on two animals prior to and following DCA administration. The methods used in these experiments were essentially those described in a recent publication from this laboratory (21).

RESULTS

1. Relationship between urine flow and urine osmolality in control and DCA-treated animals

The effects of varying degrees of mannitol diuresis on the relationship between urine flow and urine osmolality during a typical control experiment are shown in Table I. During the two preinfusion collections, the urine flow was at low levels of about 0.2 ml. per minute. Concomitantly, urine osmolality exceeded that of plasma about sevenfold as indicated by the respective osmotic U/P ratios. Both the osmolal clearance (C_{osm}) and the amount of solute-free water reabsorbed ($T^c_{H_2O}$) have small absolute values.[2] Urine flow

[2] Hypertonic urine is envisaged as consisting of two hypothetical portions: *1)* an isosmotic moiety (osmolal clearance, C_{osm}, equalling $U_{osm}V/P_{osm}$) and *2)* that amount of solute-free water which has to be abstracted from it to produce the observed hypertonicity. This amount is referred to as $T^c_{H_2O}$ and numerically equals $C_{osm}-V$. On the other hand, a hypotonic urine can be represented by an isosmotic portion (C_{osm}) and that additional amount of solute-free water which would have to be added to reduce its osmotic concentration to that observed. This latter moiety equals $V-C_{osm}$ and is referred to as C_{H_2O}. This concept as presented is that of Wesson and Anslow (10), but certain important considerations as to its application should be noted (22).

increased markedly after the 20 per cent mannitol solution was infused at increasing rates. In this experiment the peak urine flow reached 9.0 ml. per minute. As the urine flow increased, urine osmolality fell progressively but at all times remained distinctly above that of plasma as can be seen from the respective osmotic U/P ratios which exceeded unity at all urine flows. It is evident that $T^c_{H_2O}$ at first increased and then levelled off when urine flow rates exceeded about 2 ml. per minute. No significant changes in glomerular filtration rate were observed during the infusion of mannitol in this and other control experiments. The observed relationship between urine flow, osmolal clearance and $T^c_{H_2O}$ is in essential agreement with findings reported by Page and Reem (19) and others (11, 22). It should be noted that similar results were obtained when higher doses of vasopressin (80 to 340 mU priming, 80 to 500 mU per hour sustaining infusion) were given. Furthermore, we never observed an increase in $T^c_{H_2O}$ when the level of vasopressin was acutely increased by supplements of 100 and 200 mU vasopressin administered intravenously. This also is in agreement with Zak, Brun and Smith (11) indicating that hydropenia and hypertonic mannitol infusions produce maximal effects on the elaboration of an osmotically concentrated urine which cannot be enhanced by increasing the amounts of exogenous vasopressin.

The relationship between urine flow and urine osmolality was strikingly different after DCA treatment. A representative experiment is presented in Table II. Treatment with this steroid and the administration of a sodium chloride supplement resulted in a reduction of the preinfusion plasma potassium level to a subnormal value (2.5 mEq. per L). Urine osmolality at low urine flows was significantly reduced as can be seen from the low osmotic U/P ratios of 2.29 and 2.27, respectively. As urine flow increased, urine osmolality fell, and at flow rates of 4.4 ml. per minute and above, the urine formed became hypotonic (osmotic U/P ratios below 1.0). It is also apparent that the values of $T^c_{H_2O}$ decreased steadily as the urine flow increased. Despite the continuing administration of vasopressin, higher rates of mannitol diuresis enhanced urinary hypotonicity leading to a progressive rise of C_{H_2O} from values of 0.26 ml. per minute to 2.60 ml. per minute at peak urine flows. Inspection of the last column shows that glomerular filtration rates remained fairly constant throughout the course of the experiment.

Figure 1 contains results of a typical sequence of three experiments (Experiments 14, 21 and 24, performed on Dog No. 1, see Tables III and IV) showing the effects of DCA treatment and potassium repletion. Data of the respective control experiment are included. Urine flow is plotted on the abscissa and osmolal clearance (C_{osm}) on the ordinate. Solute-free water clearance (C_{H_2O}) is given by the horizontal distance between the diagonal line (isosmotic parameter) and the observed points to the right. The amount of solute-free

TABLE II

Results of an experiment showing the effect of DCA (1.0 mg. per Kg. per day for six days) on the elaboration of a concentrated urine in the hydropenic state (Experiment 20, 23 Kg. dog) *

Time	Urine flow	Urine	Plasma	Osmotic U/P ratio	C_{osm}	$T^c_{H_2O}$	C_{H_2O}	GFR
min.	*ml./min.*	*mOsm./ Kg. H_2O*	*mOsm./L.*		*ml./min.*	*ml./min.*	*ml./min.*	*ml./min.*
0		No infusion						
0– 30	0.11	687	300	2.29	0.25	0.14		
30– 50	0.10	684	302	2.27	0.25	0.15		
55	Prime: Creatinine, 1.5 Gm.; vasopressin, 50 mU; H_2O, 20 ml.							
60	Sustaining I: Infuse at 0.4 ml./min. creatinine, 7.0 Gm.; vasopressin, 400 mU; H_2O, 120 ml.							
62	Sustaining II: Infuse at increasing rates 100 Gm. mannitol; 4.5 Gm. NaCl; 500 ml. H_2O							
50– 90	0.94	496	306	1.62	1.53	0.59		
90–100	1.32	408	307	1.33	1.76	0.44		69.3
100–110	1.92	365	310	1.18	2.26	0.34		67.0
110–120	4.40	293	313	0.94	4.14		0.26	74.0
120–130	5.90	278	318	0.87	5.16		0.74	69.3
130–140	8.70	257	326	0.79	6.87		1.83	66.5
140–150	11.50	257	332	0.77	8.90		2.60	66.5

* Preinfusion plasma potassium level: 2.50 mEq. per L.

TABLE III

Osmotic diuresis during hydropenia and vasopressin administration in control and potassium repleted dogs

1	2	3	4	5	6	7	8	9	10	11	12	13
											Pitressin®	
Condition	Dog	Expt.	Urine flow (range)	Max. osmotic U/P ratio	Max. $T^c_{H_2O}$*	Regression coeff.† (slope)	Correl. coeff.‡	P_{osm} (range)	Plasma K§	GFR (mean)	Prime	Sustainin
	no.	*no.*	*ml./min.*		*ml./min.*			*mOsm./Kg. H_2O*	*mEq./L.*	*ml./min.*	*mU*	*mU/hr*
Control	1	2	0.13–11.0	4.83	2.00	0.993	0.984	293–311	3.75	81.3	40	80
Control	2	6	0.12– 7.0	5.24	1.97	0.980	0.977	296–322	3.90	51.3	40	80
Control	3	11	0.19– 5.5	4.94	2.57	1.050	0.979	281–298	3.95	82.9	40	80
Control	4	12	0.19– 9.0	7.03	4.46	0.900	0.999	302–342	3.80	71.0	40	80
Control	1	14	0.09– 9.4	5.28	2.46	0.968	0.985	304–330	3.64	59.0	40	80
Control	5	23	0.15–10.4	5.18	2.10	0.863	0.999	304–331	3.75	66.2	40	80
Control	6	31	0.09– 9.5	6.63	2.55	0.870	0.992	299–332	3.68	74.0	40	80
Control	7	32	0.10– 6.0	7.58	2.31	0.873	0.999	305–336	3.41	56.0	40	80
Control	4	13	0.05– 7.5	4.45	4.02	1.360	0.969	301–324	3.99	85.0	40	80
Control	8	36	0.08–12.6	6.39	3.40	1.170	0.991	303–321	3.91	68.0	20	44
Control	9	39	0.10– 8.8	5.71	2.40	0.910	0.959	287–302	3.66	70.7	20	44
Mean value				5.75	2.95	0.994						
Standard deviation				±1.01	±0.86	±0.152						
K-Repletion	1	9	0.12– 6.3	4.80	1.86	0.950	0.985	306–324	4.40	66.0	40	80
K-Repletion	4	22	0.10–10.2	3.99	2.16	0.968	0.987	302–322	3.62	91.6	40	80
K-Repletion	1	24	0.10– 9.6	4.94	1.33	1.018	0.955	308–336	3.37	61.2	40	80
K-Repletion	4	25	0.08– 9.1	4.00	2.04	0.941	0.989	306–324	3.69	89.7	40	80
K-Repletion	7	37	0.06– 9.2	7.46	2.65	1.080	0.990	306–345	3.89	51.9	40	80
K-Repletion	7	43	0.14–11.3	7.20	3.10	0.960	0.969	295–347	3.49	52.3	340	500
Mean value				5.25	2.19	0.986						
Standard deviation				±1.28	±0.62	±0.054						

* Since the slope of C_{osm} on V (regression coefficient) varied among animals and often deviated significantly from 1.0, we have chosen to compare maximum solute-free water reabsorption instead of selecting a value of $T^c_{H_2O}$ at any arbitrarily selected urine flow rate.

† Regression of C_{osm} on V was calculated by the method of least squares taking such points at higher urine flow rates as showed linear regression of C_{osm} on V, thus care being taken not to include points within the range of splay of the titration curve at intermediate urine flow rates (38).

‡ As pointed out by Boyarsky and Smith (38), the calculation of the correlation coefficient for linear regression of C_{osm} on V is best performed by using the linear equation $U_{osm}V/P_{osm} = aV + b$, and dividing both sides by V. Thus the two variables, U_{osm}/P_{osm} and $1/V$, are independent and, in contrast to the original linear regression of C_{osm} ($= U_{osm}V/P_{osm}$) on V, do not contain simultaneous and correlated errors by having one variable, V, occur in both terms.

§ Plasma potassium levels were measured before any infusions were given.

water reabsorbed ($T^c_{H_2O}$) from C_{osm} is shown by the horizontal distance between the isosmotic parameter and the experimental points to the left.

The experiments presented in Figure 1 illustrate two significant features. First, in the control and repletion experiment the osmolal clearance always exceeds the urine flow. It can be seen that the relationship between these two variables is such that the data generate a linear regression having a slope of approximately 1.0. In striking contrast, the slope is significantly less than 1.0 after DCA treatment. Second, it is evident that after DCA treatment a hypertonic urine was elaborated only at low urine flows. At about 5.4 ml. per minute a transition from hyper- to hypotonicity occurred. Inspection of Figure 1 also shows that these observed changes (reduction in osmolal U/P ratios, decreased $T^c_{H_2O}$, decrease in slope of C_{osm} on V) were almost completely reversible after DCA had been discontinued and oral potassium chloride supplements had been given for seven days. Essentially the same results were obtained in six similar sequences of experiments.

Figure 2 shows the effect of varying the amount of vasopressin after DCA treatment (Experiments 32, 33 and 34, performed on Dog No. 7, see Tables III and IV). In this animal, the effect of steroid administration was less marked. However, reduction of maximum osmolal U/P ratios at minimal urine flows and a decrease of $T^c_{H_2O}$ was observed. Furthermore, a small decrease in steepness of the slope of C_{osm} on V was seen. Doubling the amount of vasopressin had no significant effect on the process of urinary concen-

TABLE IV

Osmotic diuresis during hydropenia and vasopressin administration under various experimental conditions

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
													Pitressin®	
Condition	Days treated	Dog	Expt.	Urine flow (range)	Max. osmotic U/P ratio	Max. $T^*_{H_2O}$*	Max. C_{H_2O}	Regression coeff.† (slope)	Correl. coeff.‡	P_{osm} (range)	Plasma K§	GFR (mean)	Prime	Sustaining
		no.	*no.*	*ml./min.*		*ml./min.*	*ml./min.*			*mOsm./Kg. H_2O*	*mEq./L.*		*mU*	*mU/hr.*
DCA + high NaCl	7	1	5	0.10– 9.10	3.10	.99	.78	0.856	0.989	295–312	2.63	74.2	40	80
DCA + high NaCl	12	1	7	0.21– 9.40	2.18	.62	3.40	0.621	0.967	304–331	2.28	65.7	40	80
DCA + high NaCl	7	2	8	0.14– 9.20	4.28	1.42	1.37	0.702	0.998	301–339	3.18	51.2	40	80
DCA + high NaCl	7	3	15	0.08– 6.80	2.92	1.70		0.783	0.999	300–323	2.32	74.5	40	80
DCA + high NaCl	6	1	17	0.14–11.46	3.82	1.35	1.41	0.742	0.959	302–340	2.15	62.3	40	80
DCA + high NaCl	6	4	16	0.12–11.20	2.21	0.62	2.50	0.678	0.983	308–335	2.50	72.0	40	80
DCA + high NaCl	19	3	19	0.12– 3.02	1.98	0.72		0.735	0.996	308–363	2.32	53.3	40	80
DCA + high NaCl	14	4	20	0.10–11.50	2.29	0.58	2.60	0.776	0.997	300–332	2.50	71.3	40	80
DCA + high NaCl	14	1	21	0.23–13.53	2.46	1.14	2.69	0.682	0.984	309–358	2.50	50.5	40	80
DCA + high NaCl	17	7	34	0.08– 8.00	2.79	1.29		1.038	0.946	317–354	2.05	51.0	40	80
DCA + high NaCl	10	7	33	0.06– 8.64	3.18	1.40		0.872	0.997	310–354	2.43	44.8	20	40
DCA + high NaCl	24	7	35	0.07–14.7	2.67	1.20	.40	0.855	0.986	314–350	2.28	43.5	80	160
Mean value					2.90	1.08		0.777						
Standard deviation					±0.69	±0.36		±0.113						
Dietary K-Depletion	35	8	38	0.05–14.8	3.12	1.46		0.985	0.999	292–329	2.91	49.4	40	80
Dietary K-Depletion	38	8	40	0.08–11.2	3.10	1.38		0.992	0.987	296–327	2.95	52.8	80	160
Hydrocortisone + high NaCl	6	9	41	0.08–10.3	3.99	3.81		0.933	0.977	287–313	3.70	65.5	40	80
Hydrocortisone + high NaCl	13	9	42	0.08–10.2	4.53	4.45		1.048	0.948	287–310	3.36	71.5	40	80

* † ‡ § See footnotes to Table III for explanation.

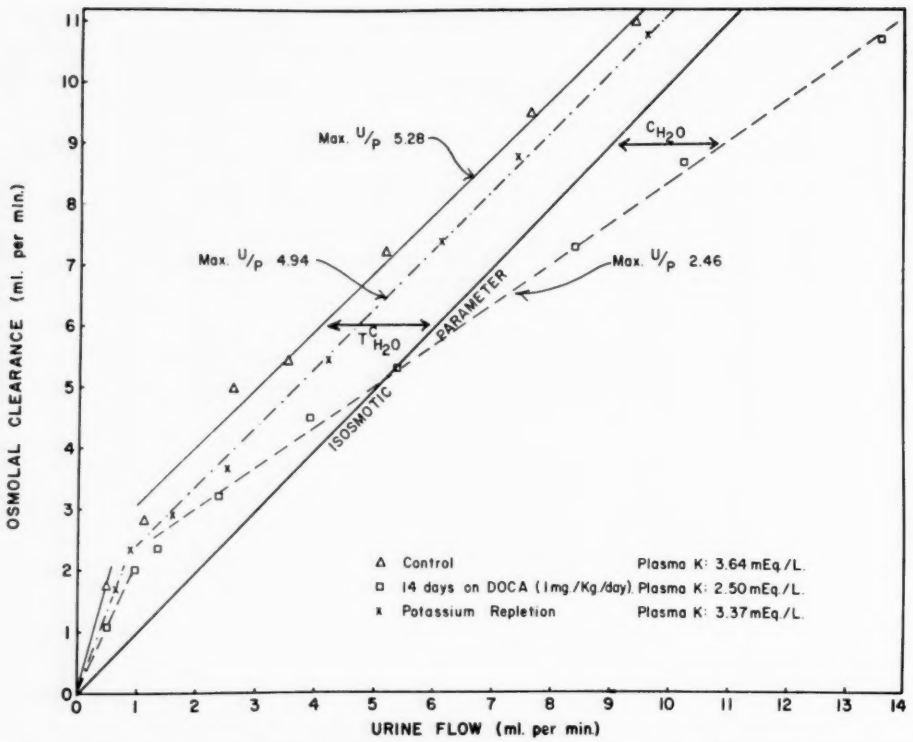


FIG. 1. THE RELATIONSHIP BETWEEN URINE FLOW AND SOLUTE EXCRETION UNDER CONTROL CONDITIONS AFTER DCA TREATMENT AND POTASSIUM REPLETION

Maximum osmotic U/P ratios were obtained at comparable low preinfusion urine flow rates.

tration. In an additional experiment, the dose of vasopressin was acutely increased in a stepwise fashion (priming infusion 80, 160, 320 mU vasopressin, sustaining infusion 160, 320, 640 mU vasopressin per hour). These additional amounts of vasopressin also were without effect on $T^c_{H_2O}$ and the slope of C_{osm} on V. This is in agreement with findings of other authors (8, 23) who observed that the polyuria developing after DCA treatment is vasopressin-resistant.

2. Relationship between urine flow and urine osmolality in animals on a high sodium chloride intake treated with hydrocortisone

In contrast to the results obtained with DCA, hydrocortisone in a dose of 7.5 mg. per Kg. per day for six days and continued in a dose of 10.0 mg. per Kg. per day for another week did not, with the exception of a small reduction of osmolal U/P ratios at low urine flows of questionable significance, result in a reduction of urinary concentrating ability. Plasma potassium levels were unchanged and filtration rate was unaltered after treatment with this steroid. Pertinent data are included in Tables III and IV.

3. Relationship between urine flow and urine osmolality after dietary potassium depletion

In one animal, a low potassium diet was given for a period of up to 38 days. Table III (Dog No. 8) and Table IV contain data on maximum osmolal U/P ratios, regression coefficients, plasma potassium levels and magnitude of glomerular filtration rate. A comparison of control values with data obtained after 35 and 38 days of feeding a low potassium diet shows that maximum osmotic U/P ratios, $T^c_{H_2O}$ and plasma potassium levels were reduced following potassium deprivation. Doub-

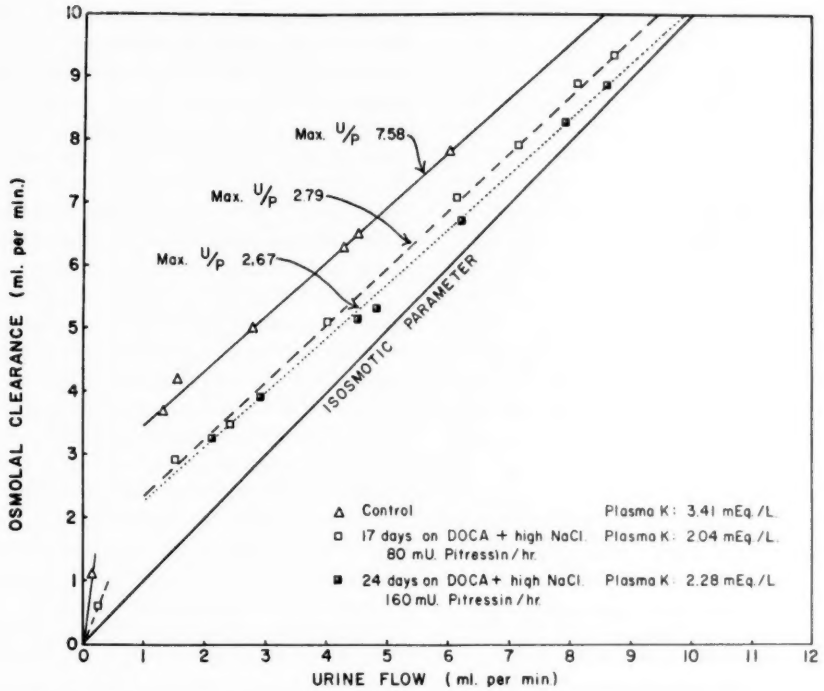


FIG. 2. THE RELATIONSHIP BETWEEN URINE FLOW AND SOLUTE EXCRETION UNDER CONTROL CONDITIONS AND AFTER DCA TREATMENT, DEMONSTRATING NO SIGNIFICANT EFFECT OF DOUBLING THE AMOUNT OF VASOPRESSIN (PITRESSIN®) INFUSED IN THIS DCA-TREATED ANIMAL

ling the dose of vasopressin had no noticeable effects on the process of forming an osmotically concentrated urine. These results are in agreement with those of other authors (4, 8) who found that dietary potassium depletion results in a significant reduction of the urinary concentrating ability which is resistant to vasopressin.

Figure 3 presents a summary of the relationship between preinfusion plasma potassium levels and osmotic U/P ratios at low urine flow rates during the several preinfusion control periods. In general, a reduced U/P ratio is found in states accompanied by a low plasma potassium level. Hollander and associates (8) also observed that the renal concentrating ability is almost linearly related to the degree of potassium depletion measured by these authors by the fall in muscle potassium. In many conditions the plasma potassium level is a poor parameter of the degree of potassium depletion. However, in our experiments it appears to be safe to assume that the reduction of the plasma potassium concentration does reflect a state of potassium deficiency. Balance studies in various species are in accord with this assumption (24, 25). It is also obvious from inspection of Figure 4 that hydrocortisone was ineffective in lowering plasma potassium levels.

A summary of pertinent data of all experiments is tabulated in Tables III and IV, permitting a comparison of individual animals under control and various experimental conditions. While most of the data are self-explanatory, several points deserve consideration. Inspection of Column 7 in Table III shows that the variation of the slope of regression lines in control experiments is considerable, ranging from 0.87 to 1.36. Inclusion of this latter value in the calculation of the mean (0.994) considerably biases this value, since in the majority of observations the slope relating urine flow to osmolal clearance deviates significantly from 1.0, being lower in 8 out of 11 control experiments. In man, values closer to unity and having a smaller

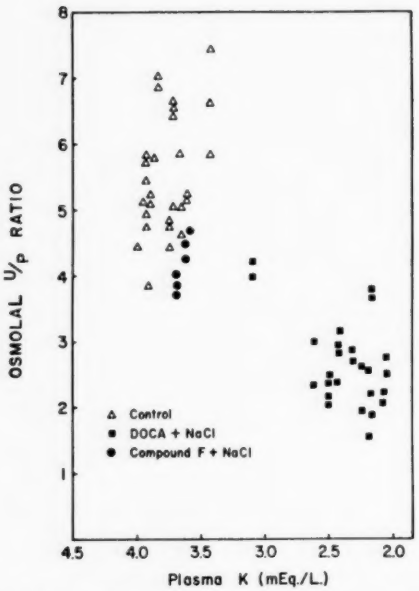


FIG. 3. THE RELATIONSHIP BETWEEN OSMOLAL U/P RATIOS AND PREINFUSION POTASSIUM LEVELS UNDER VARIOUS EXPERIMENTAL CONDITIONS

Urine flow rates ranged from 0.06 ml. per minute to 0.17 ml. per minute.

standard deviation were observed by Zak, Brun and Smith (11). It is possible that the relatively larger increase of plasma osmolality observed in our experiments (Column 9, Table III) might explain slopes consistently lower than 1.0 since a sharp rise in plasma osmolality might not be reflected immediately in the urine osmolality and result in an erroneously low osmotic U/P ratio (26). However, inspection of individual experimental protocols has not shown any clearcut relationship between rate of increase in plasma osmolality and regression coefficient. Comparison of data listed in Column 7 of Table III with those of Column 8 in Table IV indicates that in spite of similar variations in animals treated with DCA (on high NaCl intake), the regression coefficient is significantly lower in the latter, ranging from 0.621 to 1.03, with a mean of 0.777. Also, lower maximum osmotic U/P ratios (compare Column 5 in Table III with Column 6 in Table IV) and lower values of maximum $T^c_{H_2O}$ (compare Column 6 in Table III with Column 7 in Table IV) are typical for DCA-treated animals on a high sodium chloride intake. Little significance should be attached to the absolute values of maximum C_{H_2O} (Column 8, Table IV) other than indicating a significant degree of hypotonicity absent in untreated control animals over the same range of urine flows.[3] Since most of the regression coefficients (Column 9, Table IV) are smaller than 1.0, the absolute value of C_{H_2O} obviously depends on the maximum urine flow, a value showing variations among individual animals (Column 5, Table IV).

Tables III and IV permit a comparison of preinfusion plasma potassium levels in control and steroid-treated animals (Column 10, Table III and Column 12, Table IV). DCA was found to be most effective in depressing plasma potassium levels in dogs on a high sodium intake.

Finally, a striking feature in all experiments is the high coefficient of correlation for linear regression (see Column 8, Table III and Column 9, Table IV). Also, a similar high correlation coefficient, quite often in excess of 0.99, was observed in control and steroid-treated animals. Thus, in spite of large variations in the absolute values of the regression coefficients (slopes) under various experimental conditions, there is maintained a high degree of constancy of those factors which affect the relationship between urine flow and osmolal clearance, implying considerable regularity of the underlying physiological process (11).

DISCUSSION

The most important feature of our observations is that administration of DCA in hydropenic dogs results in a reduced concentrating ability of the kidney. In spite of substantial infusions of exogenous antidiuretic hormone, this effect is exaggerated at higher urine flow rates and thus is frequently associated with the formation of a urine markedly hypotonic to plasma. According to presently held views, the formation of an osmotically concentrated urine is envisaged in the following manner (27–29): Glomerular filtrate is reduced in volume during its passage along the proximal tubule by reabsorption of an isosmotic fluid.

[3] In agreement with results obtained by other authors (22), we have observed in some control experiments the excretion of a hypotonic urine during massive osmotic diuresis (urine flows above 15 ml. per minute). Therefore, we have arbitrarily limited our observations to lower flow rates.

Therefore, no change in the osmotic pressure of the tubule fluid occurs at this site. Active sodium transport out of the water-impermeable distal part of Henle's loop dilutes the tubular urine and achieves hypertonicity of the medullary interstitial fluid. In the presence of endogenous or exogenous antidiuretic hormone, the distal tubule and collecting duct is relatively water-permeable. Consequently the hypotonicity of the tubular urine is dissipated as water equilibrates with cortical interstitial fluid, resulting in the elaboration of a reduced volume of isosmotic fluid in the second part of the distal convoluted tubule. Finally, the collecting ducts, being permeable to water, permit further equilibration, this time with the hypertonic medullary interstitial fluid, rendering the final urine osmotically more concentrated than plasma.

We believe that the reduced ability of the kidney to concentrate the urine after DCA treatment is due, at least to some extent, to diminished permeability of the distal tubule and the collecting duct to water; thus less water leaves the tubule lumen in response to the osmotic gradient established by solute reabsorption. In particular it is suggested that the frequently observed hypotonicity of the urine represents a failure of the second part of the distal convolute and the collecting duct to concentrate the hypotonic fluid which emerges from Henle's loop. As urine flow increases and less time is available for the dissipation of an osmotic gradient across a membrane less permeable to water, the final urine would, in essence, approach the tonicity of the fluid within the first part of the distal tubule. The reduction of the slope of the regression line of C_{osm} on V, almost invariably observed after DCA treatment, is in accord with such an interpretation. Thus, the reduced slopes are consistent with our view that in the presence of diminished water-permeability of the distal tubular epithelium, reabsorption of water, presumed to be a passive process, does not, as usually, proceed from a hyposmotic to an isosmotic level. As urine flow increases at higher rates of solute excretion (increasing mannitol diuresis), back-diffusion of water may progressively lag behind the reabsorption of solutes, thereby preventing the attainment of an osmotic equilibrium between hyposmotic tubular urine and peritubular fluid. Abstraction of only a small amount of solute-free water from an increasingly hypotonic fluid would lead first to a reduction of $T^c_{H_2O}$ and, at higher urine flow rates, to a progressive increase in C_{H_2O}. Only at low urine flow rates would the fluid traversing the distal tubule be rendered isosmotic, since only then sufficient time would be available for the dissipation of the osmotic gradient between tubular urine and cortical interstitial fluid. Indeed, micropuncture studies by Gottschalk, Mylle, Winters and Welt (30) have shown that the osmolality of distal tubule fluid in hydropenic, potassium-depleted rats was similar to that of control animals, in spite of the fact that in the former group the urine was less concentrated. Abstraction of a reduced amount of solute-free water from an isosmotic distal tubular fluid is consistent with our results and those obtained by other authors (8, 30) showing that, although diminished, osmolal U/P ratios in excess of unity are regularly observed at low urine flows. According to this interpretation, the decrease in final water abstraction at low urine flows would be attributable to a diminished permeability of the collecting ducts to water.

A second line of evidence in favor of the collecting duct system to be involved in the functional defect of the renal concentrating mechanism is the finding of Oliver and his associates (31) that the collecting ducts are uniformly affected in potassium depleted rats. Their observations of swelling and hyperplasia of the tubular epithelium and intracellular accumulation of granules as well as marked thickening of the basement membrane might be consistent with decreased water permeability of these renal tubular structures.

While the results of our experiments can be explained by decreased permeability of the distal tubules and the collecting duct system to water, a number of alternative or additional possibilities must be considered.

First, sodium reabsorption might be diminished at some critical site within the nephron after DCA and/or potassium depletion. Failure of sodium conservation, either at the level of Henle's loop (13, 14) or the collecting ducts (32), could result in diminished hypertonicity of medullary interstitial fluid. As a consequence, diffusion of water out of the terminal part of the nephron due to a diminished osmotic concentration gradient might render the urine less concentrated.[4]

[4] A number of sodium transport mechanisms are known to be sensitive to lack of potassium (33–36). Since proxi-

Second, sodium reabsorption may be relatively enhanced following DCA administration, increasing free water and rendering the fluid within the distal tubule and collecting duct more hypotonic. Again, particularly at high urine flow rates, water reabsorption might lag sufficiently to permit excretion of hypotonic urine. While in dietary potassium depletion no increase in hypotonicity of the early distal tubule fluid has been found in micropuncture studies (30), such a possibility cannot be ruled out in steroid-treated animals. Also, sodium and chloride retention are known to occur during the development of experimental potassium depletion (4).

Third, although it is very unlikely, changes in filtration rate could have been responsible for the observed alterations in the renal concentrating process. A comparison of mean control filtration rates (Column 11, Table III) with data obtained in DCA-treated animals (Column 13, Table IV) shows that usually there was at most a moderate decrease in filtration rate in the latter group. However, a reduction in glomerular filtration rate of the magnitude observed would tend to increase urine osmolality rather than to decrease it (28, 37). To test whether nephron activity was changed following DCA administration associated with a state of potassium deficiency, glucose T_m measurements were done on two animals (Dogs No. 1 and 2) prior to and following steroid treatment. No change was observed in spite of marked and typical alterations in the urinary concentrating ability. Also, glucose titrations showed no difference in splay. Hence, we believe that alterations in filtration rate or of nephron perfusion are excluded as the factor responsible for the difference in the concentrating ability which we observed.

SUMMARY

1. Administration of DCA (1.0 mg. per Kg. per day for 6 to 24 days) to dogs on a high sodium chloride intake resulted in reduction of maximum osmotic U/P ratios at low urine flows in the hydropenic state. During mannitol diuresis and vasopressin infusion, the amount of solute-free water reabsorption ($T^c_{H_2O}$) was significantly decreased. At urine flow rates above 5 ml. per minute, the urine was frequently hypotonic. The slope of osmolal clearance on urine flow was significantly less (mean, 0.777) than in control animals (0.994). These changes were vasopressin-resistant. Plasma potassium levels were reduced, indicating potassium deficiency. Discontinuation of DCA and administration of potassium chloride supplements showed that the observed alterations were reversible.

2. Hydrocortisone (7.5 mg. per Kg. per day and 10.0 mg. per Kg. per day for periods of 6 to 13 days) did not significantly decrease maximum osmolal U/P ratios, $T^c_{H_2O}$ or the slope relating C_{osm} to V. This steroid was also ineffective in dogs on a high sodium chloride intake to reduce the plasma potassium level.

3. The data presented have been interpreted to indicate a diminished permeability of the renal epithelium of the distal tubules and collecting ducts to water after DCA and potassium depletion. However, redistribution of solute reabsorption within the nephron has not been excluded.

mal and distal sodium reabsorption show a number of essential functional differences, it is possible that the latter, involved in the establishment of considerable concentration gradients, is more sensitive to potassium deficiency.

REFERENCES

1. Ragan, C., Ferrebee, J. W., Phyfe, P., Atchley, D. W., and Loeb, R. F. Syndrome of polydipsia and polyuria induced in normal animals by desoxycorticosterone acetate. Amer. J. Physiol. 1940, **131**, 73.
2. Ferrebee, J. W., Parker, D., Carnes, W. H., Gerity, M. K., Atchley, D. W., and Loeb, R. F. Certain effects of desoxycorticosterone: the development of "diabetes insipidus" and the replacement of muscle potassium by sodium in normal dogs. Amer. J. Physiol. 1941, **135**, 230.
3. Dustan, H. P., Corcoran, A. C., and Page, I. H. Renal function in primary aldosteronism. J. clin. Invest. 1956, **35**, 1357.
4. Relman, A. S., and Schwartz, W. B. The kidney in potassium depletion. Amer. J. Med. 1958, **24**, 764.
5. Stanbury, S. W., Gowenlock, A. H., and Mahler, R. F. Interrelationship of potassium deficiency and renal disease *in* Aldosterone, A. F. Muller and C. M. O'Connor, Eds. London, Churchill, 1958, p. 155.
6. Smith, S. G., and Lasater, T. E. A diabetes insipidus-like condition produced in dogs by a potassium deficient diet. Proc. Soc. exp. Biol. (N. Y.) 1950, **74**, 427.
7. Brokaw, A. Renal hypertrophy and polydipsia in potassium-deficient rats. Amer. J. Physiol. 1953, **172**, 333.

8. Hollander, W., Jr., Winters, R. W., Williams, T. F., Bradley, J., Oliver, J., and Welt, L. G. Defect in the renal tubular reabsorption of water associated with potassium depletion in rats. Amer. J. Physiol. 1957, **189**, 557.
9. Milne, M. D., and Muehrke, R. C. Potassium deficiency and the kidney. Brit. med. Bull. 1957, **13**, 15.
10. Wesson, L. G., Jr., and Anslow, W. P., Jr. Effect of osmotic and mercurial diuresis on simultaneous water diuresis. Amer. J. Physiol. 1952, **170**, 255.
11. Zak, G. A., Brun, C., and Smith, H. W. The mechanism of formation of osmotically concentrated urine during the antidiuretic state. J. clin. Invest. 1954, **33**, 1064.
12. Brodsky, W. A., Rapoport, S., and West, C. D. The mechanism of glycosuric diuresis in diabetic man. J. clin. Invest. 1950, **29**, 1021.
13. Wirz, H. Der osmotische Druck in den corticalen Tubuli der Rattenniere. Helv. physiol. pharmacol. Acta 1956, **14**, 353.
14. Gottschalk, C. W., and Mylle, M. Micropuncture study of osmolality of renal tubular fluid in the hydropenic rat during osmotic diuresis. Fed. Proc. 1958, **17**, 58.
15. Epstein, F. H., Kleeman, C. R., Pursel, S., and Hendrikx, A. The effect of feeding protein and urea on the renal concentrating process. J. clin. Invest. 1957, **36**, 635.
16. Giebisch, G., MacLeod, M. B., and Pitts, R. F. Effect of adrenal steroids on renal tubular reabsorption of bicarbonate. Amer. J. Physiol. 1955, **183**, 377.
17. Howell, D. S., and Davis, J. O. Relationship of sodium retention to potassium excretion by the kidney during administration of desoxycorticosterone acetate to dogs. Amer. J. Physiol. 1954, **179**, 359.
18. Relman, A. S., and Schwartz, W. B. The effect of DOCA on electrolyte balance in normal man and its relation to sodium chloride intake. Yale J. Biol. Med. 1952, **24**, 540.
19. Page, L. B., and Reem, G. H. Urinary concentrating mechanism in the dog. Amer. J. Physiol. 1952, **171**, 572.
20. Peters, J. P., and Van Slyke, D. D. Quantitative Clinical Chemistry. Baltimore, Williams and Wilkins, 1943.
21. Kessler, R. H., Heidenreich, O. P. A., and Pitts, R. F. Evaluation of cell separation hypothesis of autoregulation of renal blood flow and filtration rate. Glucose titrations in normal and anemic dogs. Amer. J. Physiol. 1957, **191**, 501.
22. Orloff, J., Wagner, H. N., Jr., and Davidson, D. G. The effect of variations in solute excretion and vasopressin dosage on the excretion of water in the dog. J. clin. Invest. 1958, **37**, 458.
23. Mulinos, M. G., Spingarn, C. L., and Lojkin, M. E. Diabetes insipidus-like condition produced by small doses of desoxycorticosterone acetate in dogs. Amer. J. Physiol. 1941, **135**, 102.
24. Davis, J. O., and Howell, D. S. Comparative effects of ACTH, cortisone and DCA on renal function, electrolyte excretion and water exchange in normal dogs. Endocrinology 1953, **52**, 245.
25. Cooke, R. E., Segar, W. E., Reed, C., Etzwiler, D. D., Vita, M., Brusilow, S., and Darrow, D. C. The role of potassium in the prevention of alkalosis. Amer. J. Med. 1954, **17**, 180.
26. Welt, L. G. The influence of disease on the renal excretion of water. Yale J. Biol. Med. 1956, **29**, 299.
27. Smith, H. W. The Kidney, Structure and Function in Health and Disease. New York, Oxford University Press, 1951.
28. Berliner, R. W., Levinsky, N. G., Davidson, D. G., and Eden, M. Dilution and concentration of the urine and the action of the antidiuretic hormone. Amer. J. Med. 1958, **24**, 730.
29. Wirz, H. The location of antidiuretic action in the mammalian kidney *in* The Neurohypophysis, Proceedings of the Eighth Symposium of the Colston Research Society, H. Heller, Ed. New York, Academic Press Inc., 1957, p. 157.
30. Gottschalk, C. W., Mylle, M., Winters, R. W., and Welt, L. G. Micropuncture study of the osmolality of renal tubular fluid in potassium-depleted rats (abstract). J. clin. Invest. 1958, **37**, 898.
31. Oliver, J., MacDowell, M., Welt, L. G., Holliday, M. A., Hollander, W., Jr., Winters, R. W., Williams, T. F., and Segar, W. E. The renal lesions of electrolyte imbalance. I. The structural alterations in potassium-depleted rats. J. exp. Med. 1957, **106**, 563.
32. Hilger, H. H., Kluemper, J. D., and Ullrich, K. J. Mikroanalytische Untersuchungen ueber die Wasser-und Natriumrueckresorption aus den Sammelrohren der Säugetierniere. Pflüg. Arch. ges. Physiol. 1957, **266**, 57.
33. Huf, E. G., Doss, N. S., and Wills, J. P. Effects of metabolic inhibitors and drugs on ion transport and oxygen consumption in isolated frog skin. J. gen. Physiol. 1957, **41**, 397.
34. Glynn, I. M. Sodium and potassium movements in human red cells. J. Physiol. (Lond.) 1956, **134**, 278.
35. Keynes, R. D. The ionic fluxes in frog muscle. Proc. roy. Soc. B 1954, **142**, 359.
36. Hodgkin, A. L., and Keynes, R. D. Active transport of cations in giant axons from Sepia and Loligo. J. Physiol. (Lond.) 1955, **128**, 28.
37. Levinsky, N. G., Davidson, D. G., and Berliner, R. W. Effects of reduced glomerular filtration on urine concentration in the presence of antidiuretic hormone (abstract). J. clin. Invest. 1958, **37**, 910.
38. Boyarsky, S., and Smith, H. W. Renal concentrating operation at low urine flows. J. Urol. (Balt.) 1957, **78**, 511.

STUDIES ON A CASE OF ESSENTIAL HYPERLIPEMIA. BLOOD LIPIDS, WITH SPECIAL REFERENCE TO THE COMPOSITION AND METABOLISM OF THE SERUM GLYCERIDES BEFORE, DURING AND AFTER THE COURSE OF A VIRAL HEPATITIS

By LARS A. CARLSON and BÖRJE OLHAGEN

(From the Department of Internal Medicine, Karolinska sjukhuset, and the King Gustaf V Research Institute, Stockholm, Sweden)

(Submitted for publication September 15, 1958; accepted January 2, 1959)

The clinical course of the syndrome of essential hyperlipemia is characterized by cutaneous xanthomas, hepatosplenomegaly and recurrent attacks of abdominal pain. The diagnosis is confirmed by the milky appearance of a fasting serum sample. It is interesting to note that 100 years ago Addison and Gull (1) described xanthoma tuberosum as well as xanthoma tendinosum. Seventy-five years were to elapse, however, before Bürger and Grütze in 1932 (2) associated the symptoms and objective findings in this disease and described the first case of essential hyperlipemia. Since then more than 50 cases have been reported in the literature; from Sweden, 10 cases were presented by Malmros, Swahn and Truedsson in 1954 (3).

Bürger and Grütze (2) not only described the syndrome of essential hyperlipemia, but also made important contributions as regards the pathogenesis and therapy of the disease. By alimentary fat load they demonstrated the existence of "Dauerlipoidemi." Treatment with a low-fat diet resulted in disappearance of the cutaneous manifestations and of the hepatosplenomegaly. These findings have since been discussed and confirmed by several writers (4–7). Thannhauser and Stanley (5) fed olive oil labeled with I^{131} to patients with essential hyperlipemia and to normal subjects. They found increased concentration and prolonged retention of I^{131} in the blood of the former group. Havel (8) studied three brothers with essential hyperlipemia who had a prolonged retention of glycerides in the blood stream after a fatty meal. He was able to demonstrate an almost complete deficiency of the heparin-induced lipemia clearing factor. It was suggested that the cause of the hyperlipemia in these cases apparently was related to the metabolism of exogenous glycerides.

Schrade, Becker and Böhle (6) have, however, pointed out that completely normal blood lipid levels cannot be obtained in essential hyperlipemia by dietary means. They concluded that there is also a faulty metabolism of endogenous glycerides, a theory which has also been advanced by Schettler, Eggstein and Jobst (9).

In view of these observations it seems probable that in essential hyperlipemia there is some defect in the removal of lipids from the blood stream. The following report concerns some studies on the metabolism of the serum glycerides in one case of essential hyperlipemia including the changes in the blood lipid pattern during the course of a viral hepatitis.

MATERIAL AND METHODS

Case history. The patient was a 38 year old male without known heredity of interest. At the age of 27, xanthoma tuberosum appeared on the elbows. In 1950 a plastic operation was performed on the elbows. In 1952 he was admitted to the medical department of this hospital. Physical examination then revealed a well-nourished, apparently healthy man, with xanthoma tuberosum on the elbows, the volar aspects of the fingers, and the gluteal region. On the left Achilles tendon a xanthoma the size of a walnut was palpable. Standard analyses of blood and urine gave normal results. The serum cholesterol was 480 mg. per 100 ml.; the total lipids, 1,880 mg. per 100 ml. (gravimetric analysis); and the fasting serum had a milky appearance. The basal metabolic rate was −15 per cent. An oral glucose tolerance test was slightly pathologic. Substitution therapy was given with a thyroxin preparation (Laevoxin-Nyco), 0.1 mg. daily, which he has continued to take. He was readmitted to the hospital in November, 1956, for investigation of lipid metabolism. His only complaint was slight pain at times in the cutaneous xanthomas. Apart from these, physical examination revealed nothing pathologic. The liver and spleen were of normal size on roentgenographic examina-

TABLE I

Composition of the serum lipids and lipoproteins

		Glycerides					α-Lipoproteins	
Cholesterol	Phospho-lipids	Total	Triglyc-erides	Diglyc-erides	Monoglyc-erides	Unesterified fatty acids	Cholesterol	Phospho-lipids
mg./100 ml.	*mg./100 ml.*		*mM/L.*			*mEq./L.*	*mg./100 ml.*	*mg./100 ml.*
1,040	810	24.3	23.0	1.1	0.1	0.97	38	82

tion. The BMR was +3 per cent. The serum bilirubin was 0.3 mg. per 100 ml. Paper electrophoresis was normal. He left the hospital in December. Afterwards his blood lipids were analyzed every second or third week. He was feeling quite well until Feb. 28, 1957, when he developed pain in the epigastrium. For the first three days, the pain was rather intense, sometimes radiating up under both shoulders. The pain then disappeared and the patient complained only of occasional aching under his right costal margin. At the same time, he suffered from mild fatigue and anorexia. However, all the time he was eating fat as usual. His temperature was slightly elevated, being about 37.2° C. in the mornings. No discoloration of urine or feces had been noted. On March 12 he was hospitalized with a tentative diagnosis of a viral hepatitis. The examination revealed a slightly jaundiced man. Some palpatory tenderness was present in the upper abdomen. Otherwise, the physical examination was quite normal. Weight was 72.4 Kg. (3 months earlier, 74.7 Kg.). Laboratory findings were a leukopenia (2,300 W.B.C.), normal differential count, normal hemoglobin and slightly increased sedimentation rate. The Meulengracht index was 1:24 on March 12. The subsequent clinical course has been summarized in Table III. Treatment consisted of bed rest without dietary restrictions and injections of B-vitamins (B_1, B_2 and B_6).

Extraction and purification of blood lipids. Venous blood samples were allowed to clot for about 1 hour at room temperature. In the heparin-induced clearing experiments, heparin was added to the blood samples in amounts sufficient to inhibit the clearing reaction (10), and the plasma was extracted as soon as possible. Serum or plasma was extracted with chloroform-methanol (2:1, v/v), the extract purified by washing and then subjected to chromatography on silicic acid as described earlier (11, 12).

Glyceride determinations. The glyceride concentration was determined by estimation of glyceride glycerol (11, 12). Tri-, di- and monoglycerides were separated on silicic acid according to Borgström on a microscale (11).

Cholesterol determination. Total cholesterol was determined by a slightly modified Tschugaeff reaction on saponified samples (14).

Phospholipids. Lipid phosphorus was analyzed by wet combustion and converted to phospholipids by a factor of 25.

Unesterified fatty acids. The free fatty acids in the lipid extract were separated off by an ion exchanger according to Borgström (13), converted to methyl esters and estimated by means of the hydroxamic acid reaction (15).

Lipoprotein separation. The serum lipoproteins were separated chromatographically on glass powder (16). Only two major fractions were obtained, *viz.* the α-lipoproteins and the β-lipoproteins plus the very low density lipoproteins. Cholesterol and phospholipids were determined on chloroform-methanol extracts of the separated fractions.

In vitro clearing experiment. Partially purified post-heparin clearing factor was prepared from human plasma according to Nikkilä (17). Normal chylomicrons after a fatty meal and the patient's chylomicrons were separated by centrifugation at serum density of 25,000 × G, washed twice and recentrifuged at 25,000 × G. The chylomicrons were incubated with the clearing factor preparation at pH 7.4 in a 5 per cent albumin solution. Aliquots were pipetted off at different time intervals directly into chloroform-methanol and the glyceride concentration was estimated as described above.

Glucose tolerance test. One hundred Gm. of glucose was given in the fasting state and the blood sugar determined at half hourly intervals (18).

Liver function tests. The icteric index according to Meulengracht was estimated as a measure of the degree of jaundice (19). The upper normal limit is 1:6. Alkaline phosphatase was performed according to Buch and Buch (20), the normal value being below 9 units. The thymol test was done according to McLagan (21), a value above 4 units being pathological.

Transfusion experiment. From the patient's fasting blood plasma, with a glyceride concentration of 9.85 mM per L., 250 ml. was transferred to a healthy volunteer who had been fasting for about 16 hours and continued fasting throughout the experiment. The transfusion was given at a constant rate and was completed within 20 minutes. No untoward reaction was observed.

RESULTS

The composition of the serum lipid and lipoproteins when the patient was admitted in November, 1956, is shown in Table I. Total lipids amounted to about 4 per cent. Cholesterol and phospholipids were increased about four times above normal levels, while the glycerides were increased about 20-fold. The amount of lower glycerides was higher than normal, but the percentage composi-

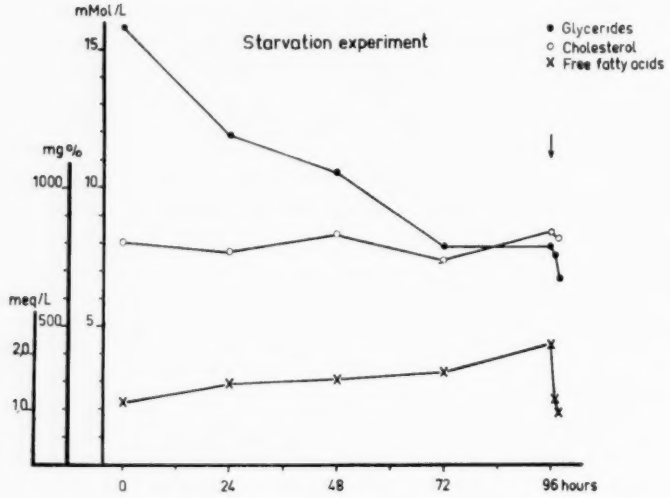


FIG. 1. BLOOD LIPIDS DURING STARVATION

Glyceride concentration is given in mM per L.; cholesterol, in mg. per 100 ml.; and free fatty acids, in mEq. per L.

tion showed no major deviation from the normal (11). The content of unesterified fatty acids was slightly above normal level, which in 10 normal persons was found to be 0.69 ± 0.10 mEq. per L. with the present technique (15). The α-lipoproteins, on the other hand, were present in normal amounts and exhibited a normal cholesterol/ phospholipid ratio. In the patient's available relatives (father and father's sister) the serum lipids were within normal range.

Starvation experiment. The patient starved for 96 hours, ingesting only mineral water and vitamins. The variations of the serum lipids during the starvation period are presented in Figure 1. The experiment was terminated by the oral administration of 100 Gm. of glucose (indicated by the arrow in Figure 1). Within two hours after the administration of glucose, the unesterified

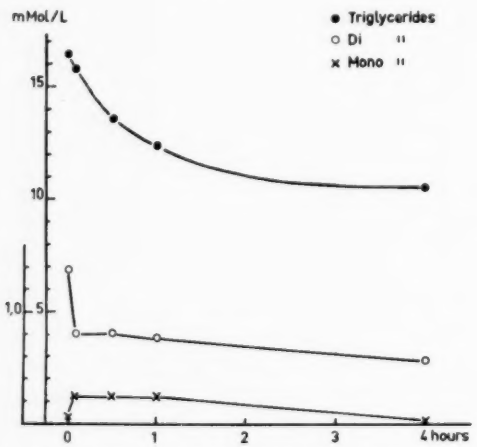


FIG. 2. RESPONSE OF THE SERUM GLYCERIDES TO A SINGLE INTRAVENOUS HEPARIN INJECTION

The partial glycerides are referred to the enlarged scale.

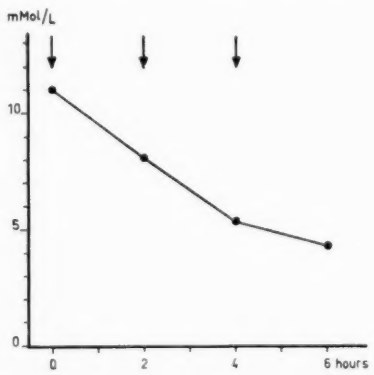


FIG. 3. RESPONSE OF THE SERUM GLYCERIDES TO REPEATED INTRAVENOUS HEPARIN INJECTIONS

Fifty mg. of heparin was given at the points indicated by arrows.

fatty acids had diminished from 2.0 to 0.9 mEq. per L. and the glycerides from 8 to 6 mM per L.

Heparin tests. These experiments were performed in two ways. First, the effect of a single intravenous injection of 50 mg. of heparin was studied by analysis of the concentration of tri-, di- and monoglycerides at various time intervals (Figure 2), and second, the concentration of total serum glycerides was followed after repeated intravenous injection of 50 mg. of heparin (Figure 3).

In vitro clearing experiment. The results obtained when normal chylomicrons and chylomicrons isolated from the patient's serum were incubated with partly purified postheparin clearing factor are given in Figure 4. It is quite evident that the patient's chylomicrons were not as readily attacked by the clearing factor preparation as were normal chylomcirons. Addition of more albumin did not influence the rate of hydrolysis.

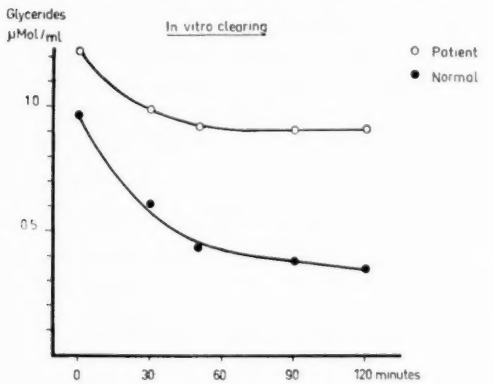


FIG. 4. *IN VITRO* CLEARING OF THE PATIENT'S CHYLOMICRONS AND NORMAL CHYLOMICRONS UPON INCUBATION WITH PARTLY PURIFIED POSTHEPARIN CLEARING FACTOR

Transfusion experiment. Figure 5 shows the glyceride concentration in the recipient's plasma during the transfusion experiment. To permit study of the kinetics of glyceride removal from the blood stream, the values were plotted on a logarithmic scale (Figure 6). The resulting curve was resolved into two exponential functions, number one being extrapolated from the curve best fitting the experimental points. The half-lives for the two functions were about 1,100 and 30 minutes, respectively.

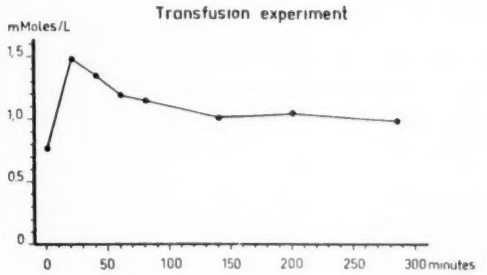


FIG. 5. SERUM GLYCERIDES IN A NORMAL HEALTHY ADULT AFTER TRANSFUSION OF PLASMA FROM THE HYPERLIPEMIC SUBJECT

The transfusion began at time 0 and ended at 20 minutes.

Glucose tolerance test. The blood glucose response to oral glucose is given in Table II.

Blood lipids during viral hepatitis. The clinical course of the hepatitis has been summarized in Table III. The serum lipids during the course of, and the recovery from, the hepatitis can be followed in Figure 7. The *fasting serum sample on admission (March 12) was almost clear* in contrast to the earlier creamy appearance of fasting specimens. It is of interest to point out that

TABLE II

Oral glucose tolerance test

Time*	8:00	8:30	9:00	9:30	10:00	10:30
Blood sugar (mg./100 ml.)	105	248	265	235	140	96

* 100 Gm. of glucose was given at 8:00.

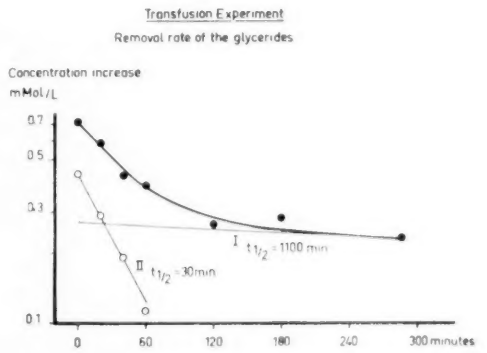


FIG. 6. SEMILOGARITHMIC DIAGRAM FROM THE TRANSFUSION EXPERIMENT (FIGURE 5)

The transfusion was finished at time 0. The concentration was calculated from the pretransfusion level.

TABLE III

Clinical course and some laboratory data during the course of a viral hepatitis

Date	12/3	13/3	20/3	27/3	3/4	10/4	29/4	27/5
General appearance		Jaundiced, tired	Jaundiced, tired	Improved, not so tired	Still more improved, out of bed 2 hr. a day	Only slightly tired	Has left the hospital (13/4)	Excellent
Weight (*Kg.*)	72.4		72.0	72.5	74.1	73.1		
Morning temperature (° *C.*)		37.1	37.0	37.0	36.6	36.6		
Meulengracht index (Normal, <1:6)	1:24	1:20	1:20	1:9	1:8	1:8	1:6	1:4
Thymol test units		2	6	6	8	8	5	4
Alkaline phosphatase, units		11	13	11	14	12	4	5

the blood lipids had already started to decline on February 18, 10 days before any subjective symptoms had appeared. The percentage composition of partial glycerides on March 12 was normal, and the concentration of unesterified fatty acids was 0.94 mEq. per L. No clearing activity could be demonstrated in the patient's blood upon incubation of his plasma with chylomicrons. During the incubation studies no changes in optical density or in unesterified fatty acid concentration were observed.

The effect of an alimentary fat load was studied on two occasions during the illness. The fat was given in the form of cream containing 40 per cent fat. The first fat load was given after three days at the hospital when the blood lipids were still decreasing. The second fat load study was performed after one month at the hospital when the blood lipids were tending to increase. Both tests gave qualitatively similar results, as shown in Figure 8.

DISCUSSION

The typical clinical features in this case were the cutaneous xanthomas and the grossly elevated content of glycerides in fasting serum samples. The fact that the concentration of unesterified fatty acids was slightly elevated is difficult to evaluate

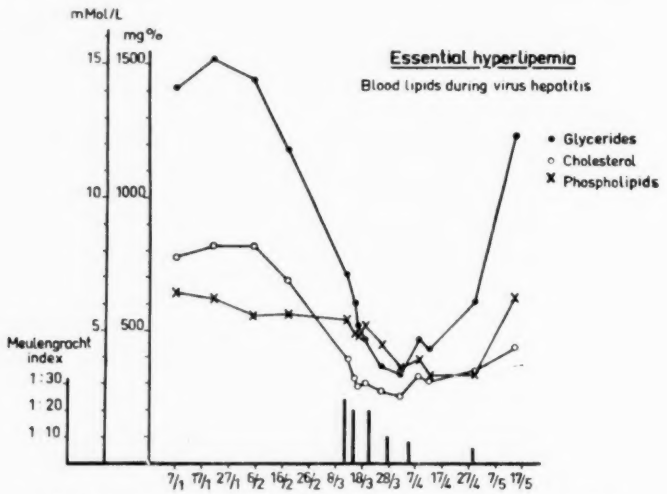


FIG. 7. BLOOD LIPIDS AND MEULENGRACHT INDEX DURING VIRAL HEPATITIS

Glyceride concentration is expressed in mM per L.; cholesterol and phospholipids, in mg. per 100 ml.

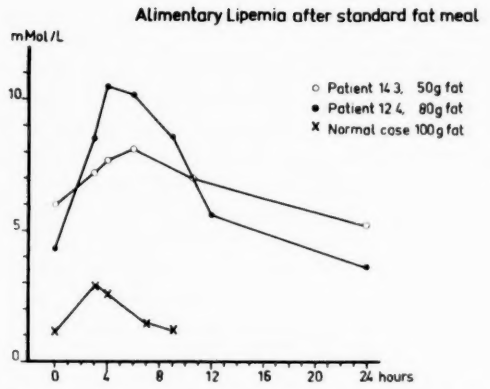


FIG. 8. SERUM GLYCERIDES AFTER A STANDARD FAT MEAL IN THE PATIENT AT TWO DIFFERENT TIMES AND IN A NORMAL SUBJECT

in view of the lability of this fraction. *In vitro* incubation of the patient's serum did not give rise to any lipolysis of significance. Normal levels of unesterified fatty acids in essential hyperlipemia were reported by Dole (22). The α-lipoprotein concentration was normal, in contrast to the findings of Havel and Gordon (23).

Although the glycerides decreased during the three first days of starvation, the level was not reduced below 8 mM per L. On the other hand, normal subjects starving for three days showed an increase in the serum glycerides which, however, did not exceed 1.5 mM per L. (11). Havel and Gordon (23) studied a case of essential hyperlipemia which, during starvation with an R.Q. of 0.73, had an essentially normal blood lipid pattern. Their interpretation was that the *endogenous* lipid metabolism was operating in the normal way in this patient. The findings during starvation, suggest that, in our patient, there was a faulty metabolism of endogenous glycerides.

The increase of unesterified fatty acids during the starvation period was similar to that reported for normal individuals during starvation (24). The immediate fall in this fraction after glucose feeding is on a par with earlier findings in normal subjects (22, 25).

Heparin administration in this case of essential hyperlipemia resulted in a reduction of the glyceride concentration. Several writers have noted this type of response in essential hyperlipemia (26–30). Others, however, have reported absence of heparin response (8).

After a single heparin dose in our case, the reaction of the partial glycerides was the same as in normals (31). The principal feature was a decrease of triglycerides and an increase of monoglycerides. This glyceride pattern during lipemia clearing can be explained if the clearing factor primarily attacks the α-ester bond in the glyceride molecule (32).

A considerably decreased plasma glyceride concentration was obtained when heparin was administered three times at intervals of two hours. The glyceride level then fell by about 60 per cent in six hours.

These heparin experiments showed that the patient's capacity to produce clearing-factor activity in his blood was similar to that of normal persons. Further, the mechanism for removing fatty acids—principally serum albumin—must have been intact, since otherwise an accumulation of these products of hydrolysis would necessarily have retarded, and finally inhibited, the clearing reaction.

The *in vitro* clearing experiment indicated that the very low density material isolated from the patient was not as readily hydrolyzed by the clearing factor preparation as were normal chylomicrons. This has been shown earlier in essential hyperlipemia from this laboratory (11). The behavior is, however, once again in contrast to that found in Havel's patients, where the chylomicrons served as an excellent substrate for the lipoprotein lipase (8).

When the glyceride increase in the transfusion experiment is plotted on a semilogarithmic scale versus time, the curve could be resolved into two exponential functions. This procedure requires the assumption that the endogenous glyceride level remained unchanged in the recipient during the experiment. In a study of the glycerides during starvation in normals, no increase was found before 39 hours (11) nor were any consistent changes observed in repeated blood samples during the early phase of starvation after an overnight fast (33). These findings seem to justify the above assumption.

The behavior of the glycerides was strikingly different from what has been reported to occur when chylomicrons are transfused to dogs (34)

and rats (35). In these experiments the elimination from the plasma was a simple exponential function of time. Havel observed the same first order reaction for the elimination of the glycerides when a normal man was transfused with plasma from one of his patients with essential hyperlipemia (8). Eder transfused chylous ascites to a patient with carcinoma and found that the removal was first rapid, with a half-life of 15 minutes, but that there was a second and slower component with a half-life of two hours (36). The half-life of the first phase in our case is of about the same magnitude as that reported earlier in animals (34, 35) and man (8, 36). Any second phase of the magnitude observed here has not been seen when purified chylomicrons have been studied nor when hyperlipemic plasma was given to a normal individual (8). In the case of chylous ascites, on the other hand, a second phase was observed. However, the half-life for the second phase in our case was much longer.

The second slope could result either from a distribution in a second pool or from a heterogeneity of the glycerides. The magnitude of this second component of the curve, when compared to available data on the removal of chylomicrons, makes the latter view appear the most probable. If so, this glyceride fraction consisted of about 30 to 40 per cent of the transfused amount of glycerides.

The slightly pathologic glucose tolerance curve is of great interest. Similar findings have been reported earlier in essential hyperlipemia and compiled by Lever, Smith and Hurley (37). Adlersberg and Wang have described the syndrome of essential hyperlipemia, mild diabetes mellitus and severe vascular damage (38). There was, however, no evidence in this patient of any vascular disease.

The decreased glyceride level during the hepatitis is to be ascribed to a change in the metabolism of either *exogenous* or *endogenous* glycerides or both. There seems to be no reason to assume a decreased glyceride level due to a diminished fat intake as far as could be judged from the history and the eating habits during the hospital stay. Furthermore the *decrease was already observed on February 18, 10 days before any subjective complaints*. Similarly, a decreased intestinal absorption of fat of such a magnitude as to influence the blood glyceride level seems rather unlikely. It is known from studies in animals (39) and man (40) that in the total absence of bile 60 to 70 per cent of the alimentary fat is absorbed. Furthermore, the amount of fat in feces during hepatitis is directly correlated with the bilirubinemia (41, 42), and in our case the glycerides decreased before the jaundiced stage and continued to decrease when the bilirubinemia leveled off. Interestingly, no significant rise in blood lipids during fat absorption was observed in dogs with bile fistula (43). In bile fistula rats Borgström found that, at the most, 25 per cent of the absorbed fat was transported via the thoracic duct lymph (44). However, a significant rise in the serum glycerides was seen in our patient after a fatty meal during the hepatitis. Thus, the liver disease does not seem to have exerted any significant influence on the resorptive phase of the metabolism of exogenous glycerides. It has not been possible to quantify the elimination phase, although the resorption curves in Figure 8 suggest that this phase was quantitatively of the same magnitude when the glycerides were dropping sharply as when they had started to increase. Furthermore, this phase definitely differed from what is seen normally, with a greater increase and a prolonged retention of the glycerides in the blood stream, indicative of a decreased removal rate of the alimentary chylomicrons in this patient even when the plasma lipids were at their lowest value during the hepatitis.

The possibility that the metabolism of endogenously derived glycerides was altered is suggested by *1*) the fact that in spite of the greatly decreased glyceride level no major influence was observed on the metabolism of exogenous glycerides, and *2*) the close parallelism between the cholesterol and glyceride levels during the disease. The blood cholesterol is known to be synthesized in the liver and the above-mentioned close parallelism between the two types of lipids might suggest that the liver damage *per se* had caused a decreased synthesis of triglyceride-rich lipoproteins. It is of interest in this connection that a decrease of the S_f 100 to 400 classes of lipoproteins, known to be very rich in triglyceride (45), has been reported in hepatitis (46) although the total level of glycerides seemingly is not decreased (47).

The findings in this case of essential hyperlipemia have indicated that the lipoprotein lipase

system (lipemia clearing factor system) is intact upon heparin administration while on the other hand the metabolism and transport of endogenously derived glycerides is definitely changed from the normal state. This is of great practical, as well as theoretical, interest in view of the fact that cases of essential hyperlipemia with defective lipoprotein lipase system and normal metabolism of endogenous glycerides have been reported (8, 23).

SUMMARY

A case of essential hyperlipemia was studied with special reference to the serum glycerides. The main findings were as follows.

1. The serum glycerides were greatly increased. The percentage composition of tri-, di- and monoglycerides was normal. Total cholesterol and phospholipids were moderately elevated and the unesterified fatty acids slightly increased.

2. Starvation for four days reduced the serum glycerides and increased the unesterified fatty acids. The glyceride level was, however, still 10 times above the normal value.

3. Heparin administration decreased the glyceride level. The response as regards partial glycerides was qualitatively normal.

4. *In vitro* incubation of the isolated, very low density lipoproteins with partly purified postheparin clearing factor resulted in an abnormally slow hydrolysis of the triglycerides.

5. Transfusion of the patient's plasma to a normal recipient revealed at least two exponential functions for the removal of the glycerides with half-lives of about 30 and 1,100 minutes.

6. During a viral hepatitis, the patient's milky plasma became almost clear. The principal findings in the blood lipid pattern during the hepatitis was a simultaneous, pronounced decrease of cholesterol and glycerides starting before any subjective symptoms had appeared. After healing of the hepatitis, the blood lipids started to increase.

REFERENCES

1. Addison, T., and Gull, W. Guy's Hosp. Rep. 1857, **7**, 265. (Cited *in* Harris-Jones, J. N., Jones, E. G., and Wells, P. G. Xanthomatosis and essential hypercholesterolaemia. Lancet 1957, **1**, 855.)
2. Bürger, M., and Grütze, O. Über hepatosplenomegale Lipoidose mit xanthomatösen Veränderungen in Haut und Schleimhaut. Arch. Derm. Syph. (Berl.) 1932, **166**, 542.
3. Malmros, H., Swahn, B., and Truedsson, E. Essential hyperlipaemia. Acta med. scand. 1954, **149**, 91.
4. Holt, L. E., Jr., Aylward, F. X., and Timbres, H. G. Idiopathic familial lipemia. Bull. Johns Hopk. Hosp. 1939, **64**, 279.
5. Thannhauser, S. J., and Stanley, M. M. Serum fat curves following administration of I^{131} labeled neutral fat to normal subjects and those with idiopathic hyperlipemia. Trans. Ass. Amer. Phycns 1949, **62**, 245.
6. Schrade, W., Becker, G., and Böhle, E. Das Krankheitsbild der idiopatischen Hyperlipidämie. Dtsch. Arch. klin. Med. 1954, **201**, 344.
7. Martt, J. M., and Connor, W. E. Idiopathic hyperlipemia associated with coronary atherosclerosis. Arch. intern. Med. 1956, **97**, 492.
8. Havel, R. J. Evidence for the participation of lipoprotein lipase in the transport of chylomicrons *in* Proc. Third int. Conf. on Biochemical Problems of Lipids (Brussels, 1956), p. 265.
9. Schettler, G., Eggstein, M., and Jobst, H. Die essentielle Hyperlipämie. Dtsch. med. Wschr. 1958, **83**, 1.
10. Brown, R. K., Boyle, E., and Anfinsen, C. B. The enzymatic transformation of lipoproteins. J. biol. Chem. 1953, **204**, 423.
11. Carlson, L. A., and Wadström, L. B. On the occurrence of tri-, di- and monoglycerides in human serum *in* Proc. Third int. Conf. on Biochemical Problems of Lipids (Brussels, 1956), p. 123.
12. Carlson, L. A., and Wadström, L. B. Determination of glycerides in blood serums. Clin. chim. Acta 1959, **4**, 197.
13. Borgström, B. Investigation on lipid separation methods. Separation of cholesterol esters, glycerides and free fatty acids. Acta physiol. scand. 1952, **25**, 111.
14. Hanel, H. K., and Dam, H. Determination of small amounts of total cholesterol by the Tschugaeff reaction with a note on the determination of lathosterol. Acta chem. scand. 1955, **9**, 677.
15. Carlson, L. A., and Wadström, L. B. A colorimetric method of determining unesterified fatty acids in plasma. Scand. J. clin. Lab. Invest. 1958, **10**, 407.
16. Carlson, L. A. Chromatographic separation of serum lipoproteins on glass powder columns *in* Proc. Third int. Conf. on Biochemical Problems of Lipids (Brussels, 1956), p. 113.
17. Nikkilä, E. Studies on the lipid-protein relationships in normal and pathological sera and the effect of heparin on serum lipoproteins. Scand. J. clin. Lab. Invest. 1953, **5**, Suppl. 8.
18. Reinhold, J. G. Glucose *in* Standard Methods of Clinical Chemistry, Amer. Ass. of Clin. Chemists, M. Reiner, Ed. New York, Academic Press Inc., 1953, vol. 1, p. 65.
19. Meulengracht, E. J. Studier over den kroniske hereditaere haemolytiske ikterus (thesis). Copenhagen, 1918.

20. Buch, I., and Buch, H. An improved King and Armstrong method for the determination of phosphatase activity in blood serum. Acta med. scand. 1939, **101**, 211.

21. McLagan, N. F. The thymol turbidity test; new indicator of liver dysfunction. Brit. J. exp. Path. 1944, **25**, 234.

22. Dole, V. P. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. J. clin. Invest. 1956, **35**, 150.

23. Havel, R. J., and Gordon, R. S., Jr. An enzymatic defect associated with altered lipoprotein metabolism in an individual with idiopathic hyperlipemia (abstract). Circulation 1955, **12**, 485.

24. Laurell, S. Plasma free fatty acids in diabetic acidosis and starvation. Scand. J. clin. Lab. Invest. 1956, **8**, 81.

25. Gordon, R. S., Jr. Unesterified fatty acid in human blood plasma. II. The transport function of unesterified fatty acid. J. clin. Invest. 1957, **37**, 810.

26. Van Eck, W. F., Peters, J. P., and Man, E. B. Significance of lactescence in blood serum. Metabolism 1952, **1**, 383.

27. Klatskin, G., and Gordon, M. Relationship between relapsing pancreatitis and essential hyperlipemia. Amer. J. Med. 1952, **12**, 3.

28. Joyner, C. R., Jr. Essential hyperlipemia. Ann. intern. Med. 1953, **38**, 759.

29. Herzstein, J., Wang, C. I., and Adlersberg, D. Effect of heparin on plasma lipid partition in man: Studies in normal persons and in patients with coronary atherosclerosis, nephrosis and primary hyperlipemia. Ann. intern. Med. 1954, **40**, 290.

30. Lever, W. F., Smith, P. A., and Hurley, N. A. Idiopathic hyperlipemia and primary hypercholesteremic xanthomatosis. II. Effects of intravenously administered heparin on the plasma proteins and lipids. J. invest. Derm. 1954, **22**, 71.

31. Carlson, L. A., and Wadström, L. B. Studies on the glycerides during the clearing reaction. Clin. chim. Acta 1957, **2**, 9.

32. Borgström, B., and Carlson, L. A. On the mechanism of the lipolytic action of the lipaemia-clearing factor. Biochim. biophys. Acta 1957, **24**, 638.

33. Havel, R. J. Early effects of fasting and of carbohydrate ingestion on lipids and lipoproteins of serum in man. J. clin. Invest. 1957, **36**, 855.

34. Havel, R. J., and Fredrickson, D. S. The metabolism of chylomicra. I. The removal of palmitic acid-1-C^{14} labeled chylomicra from dog plasma. J. clin. Invest. 1956, **35**, 1025.

35. French, J. E., Morris, B., and Robinson, D. S. Observations on the removal of chylomicra from the blood *in* Proc. Third int. Conf. on Biochemical Problems of Lipids (Brussels, 1956), p. 323.

36. Eder, H. A. The lipoproteins of human serum. Amer. J. Med. 1957, **23**, 269.

37. Lever, W. F., Smith, P. A. J., and Hurley, N. A. Idiopathic hyperlipemic and primary hypercholesterolemic xanthomatosis. I. Clinical data and analysis of the plasma lipids. J. invest. Derm. 1954, **22**, 33.

38. Adlersberg, D., and Wang, C. I. Syndrome of idiopathic hyperlipemia, mild diabetes mellitus, and severe vascular damage. Diabetes 1955, **4**, 210.

39. Munk, I. Über die Resorption von Fetten und festen Fettsäuren nach Ausschluss der Galle vom Darmkanal. Virchows Arch. path. Anat. 1890, **122**, 302.

40. Shapiro, A., Koster, H., Rittenberg, D., and Schoenheimer, R. The origin of fecal fat in the absence of bile, studied with deuterium as an indicator. Amer. J. Physiol. 1936, **117**, 525.

41. Gross, J. B., Comfort, M. W., Wollaeger, E. E., and Power, M. H. Total solids, fat and nitrogen in the feces. V. A study of patients with primary parenchymatous hepatic disease. Gastroenterology 1950, **16**, 140.

42. Colwell, A. R., Jr. Fecal fat excretion in patients with jaundice due to viral hepatitis. Gastroenterology 1957, **33**, 591.

43. Crandall, L. A., Jr., and Ivy, H. B. Bile salts and fat absorption (abstract). Amer. J. Physiol. 1940, **129**, P341.

44. Borgström, B. On the mechanism of the intestinal fat absorption. V. The effect of bile diversion on fat absorption in the rat. Acta physiol. scand. 1953, **28**, 279.

45. Bragdon, J. H., Havel, R. J., and Boyle, E. Human serum lipoproteins. I. Chemical composition of four fractions. J. Lab. clin. Med. 1956, **48**, 36.

46. Pierce, F. T., Jr., Kimmel, J. R., and Burns, T. W. Lipoproteins in infectious and serum hepatitis. Metabolism 1954, **3**, 228.

47. Albrink, M. J., Man, E. B., and Peters, J. P. Serum lipids in infectious hepatitis and obstructive jaundice. J. clin. Invest. 1950, **29**, 781.

COMPARATIVE STUDIES ON THE HALF-LIFE OF I^{131}-LABELED ALBUMINS AND NONRADIOACTIVE HUMAN SERUM ALBUMIN IN A CASE OF ANALBUMINEMIA

By HERMANN BENNHOLD and EKKEHARD KALLEE

(From the Medizinische Universitätsklinik, Tübingen, Germany)

(Submitted for publication February 20, 1958; accepted January 8, 1959)

Hypoalbuminemia occurs frequently in the course of wasting disease but never progresses to a condition of complete analbuminemia. The latter, in fact, is unknown in pathology. Analbuminemia, however, has been described as a benign congenital syndrome occurring in siblings (1–3). The absence of serious pathological changes in these two cases is striking and makes it appear likely that this form of benign analbuminemia belongs to the realm of congenital aberration of protein metabolism.

The absence of circulating albumin creates a unique basal condition which makes it easy to follow the fate of infused albumin by more than one method. It was decided, therefore, to study the rate of disappearance of albumin in one of the siblings (a female) and to use for this purpose both I^{131}-labeled albumin and a commercial human albumin. The rate of disappearance of the former was obtained by measuring the decay of radioactivity in the usual manner and of the latter by means of paper electrophoresis and immunological tests.

METHODS AND MATERIALS

1. Infusions of human serum albumin. During the three months preceding the first injection of human serum albumin in 1953, numerous routine clinical and biochemical tests were made (1–5). On June 10, 1953, 50 Gm. of commercial human serum albumin (Behringwerke, Marburg) [1] in 250 ml. of saline solution was injected intravenously. On the day of the injection hemoglobin was 84 per cent (or 13.5 Gm. per 100 ml.) and hematocrit values were 34 to 38 per cent. Preliminary intracutaneous tests with albumin had shown no signs of an allergic reaction. At intervals after the intravenous injection of albumin, further blood samples were taken and the tests repeated.

Further infusions of human serum albumin were made on March 30, 1954 (202 Gm. within 11 days), on Feb. 25, 1955 (150 Gm., 7 days), on Jan. 20, 1956 (200 Gm., 9 days), and on May 12 and 13, 1957 (205 Gm., 11 and 4 hours). All these infusions were tolerated well.

2. I^{131}-albumin. a) To 10 mc. of "carrier-free" I^{131} (containing 10 to 20 μg. of NaI) was added 0.015 ml. of a KIO_3 solution containing 600 μg. of KIO_3 per ml. After acidifying this mixture with 0.1 ml. of 2 N H_2SO_4, the liberated I^{131}_2 was taken up in 0.25 ml. CCl_4, and the aqueous phase was once more extracted with 0.2 ml. of CCl_4. This solution of I^{131}_2 was added to 30 mg. of human serum albumin dissolved in 0.3 ml. of 1.25 per cent Na_2CO_3 (6). The layers of both CCl_4 and albumin solution were gently mixed by slowly rotating the glass-stoppered test tube for five minutes at room temperature until the pink color of the CCl_4 layer had disappeared. The iodinated albumin solution was allowed to stand 60 more minutes; it was then carefully brought to approximate neutrality by adding 0.1 ml. of N-acetic acid. Meanwhile, a sterile Seitz microfilter was desorbed (*i.e.*, inactivated) by use of 1.0 ml. of the analbuminemic subject's serum. Then the I^{131}-albumin solution was filtered and the filter twice washed with serum portions of 0.5 ml. each. The mixture of I^{131}-albumin and serum was transferred to a dialysis bag and dialyzed six days against frequently changed 200 ml. portions of Ringer solution; the first dialyzing solution had 0.1 per cent KI added to it. At the end of the dialysis period, radioautograms of paper electrophoresis strips showed no trace of iodide, *i.e.*, less than 0.1 per cent of the protein radioactivity, nor did the protein solution contain any traces of I^{131}-tagged globulins. The total yield was 1.25 mc., *i.e.*, 12.5 per cent of the originally used 10 mc. of NaI^{131}.

This I^{131}-albumin was used for the first experiment on Feb. 22, 1954.

b) For the second experiment on May 10, 1957, we used the commercially produced I^{131}-labeled albumin, RISA® (Abbott).

c) On July 18, 1958, jet iodinated I^{131}-albumin was kindly prepared by Dr. A. S. McFarlane by the following procedure. To 40 mg. recrystallized, pasteurized human albumin (Behringwerke, Marburg) in 0.8 ml. of 0.05 M glycine buffer, pH 4.5, was added free inactive iodine prepared by slightly acidifying a balanced mixture of sodium iodide and iodate. This was added dropwise until the color of free iodine persisted, indicating that the free sulphydryl groups had been oxidized. Iodide formed in this reaction and excess iodine were removed by passing the solution through a column (25×7 mm.) of Deacidite FF resin (Permutit Co. London, Ltd.) in the

[1] This albumin was prepared without crystallization and was pasteurized in the usual manner.

chloride form. The pH of the effluent was adjusted to 9.0 by adding N NaOH and the solution taken up in a capillary pipette.

The labeling solution was now prepared by taking 0.30 ml. of a stock aqueous solution of iodine monochloride containing 0.42 mg. iodine per ml. 0.01 M HCl and M NaCl and injecting into it 0.7 ml. of 0.05 M glycine buffer, pH 9.0. This colorless solution, which effectively contains all its iodine as hypoiodite ion, now had 2.0 mc. of carrier-free I^{131} added to it. The latter solution, which must be free of reducing agent, is obtained by condensing I^{131}_2 vapor into 0.01 N NaOH and is obtainable from the Radiochemical Centre, Amersham.

The mixture was taken up in another capillary pipette and the contents of both pipettes discharged under pressure so that the fluid jets impinged on a common surface where turbulent mixing occurred. The mixture was passed through a second Deacidite column and the effluent, containing 1.2 mc. of iodine distributed at a mean level of 0.80 atom per molecule of albumin, then received 200 mg. of unlabeled albumin to reduce radiation damage during storage. Sterile filtration followed through a Seitz pad and an aliquot of the filtrate was used for injection. One and four-tenths per cent of the activity in it was nonprotein-bound as measured by trichloroacetic acid precipitation.

3) Counting techniques. a) In 1954, we counted the serum radioactivities in a gas-filled Geiger fluid-counter tube (Zentralwerkstatt, Göttingen). In each experiment we counted at least 10,000 impulses. The counter tube had a sensitivity of 11,000 cpm per μc. in a volume of 10 ml. and above a background of 28 to 30 cpm.

b) In 1957 and 1958, measurements were made in a well-type scintillation counter (Frieseke & Hoepfner, Erlangen-Bruck). This has a sensitivity of 750,000 cpm per μc. I^{131} in 3 ml., above a background of 200 cpm. We counted the 3 ml. samples in Fiolax® test tubes (Schott & Gen., Mainz).

4) Immunological and electrophoretic determinations. The immunological estimations of the albumin content of the patient's serum in 1953–54 were performed by Dr. F. Lohss after the method of Heidelberger and Kendall (7) using a rabbit antiserum with a high specificity against human albumin. Until 1956, the routine paper electrophoresis in this clinic was done by Dr. E. Roth, later by Dr. G. Scheurlen. Protein was estimated by the

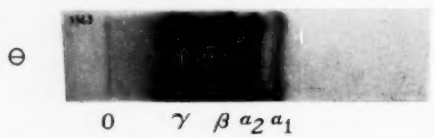


FIG. 1 A. PAPER ELECTROPHORESIS STRIP OF THE ANALBUMINEMIC SUBJECT'S SERUM ON APRIL 10, 1953, PRIOR TO THE FIRST INFUSION OF HUMAN ALBUMIN

Seven-thousandths ml. of serum was put onto the start line. No traces of albumin are visible on this strip ○ indicates start line; ⊕, anode; and ⊖, cathode.

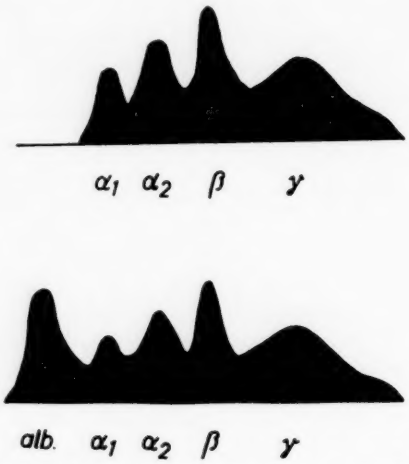


FIG. 1 B. PAPER ELECTROPHEROGRAMS OF THE SERUM OF THE SAME SUBJECT BEFORE (TOP) AND AFTER (BOTTOM) THE FIRST INJECTION OF 50 GM. OF ALBUMIN ON JUNE 11, 1953

$CuSO_4$ density method (8), and by Kjeldahl nitrogen determinations. Paper electrophoresis was carried out using the Grassmann apparatus with sodium veronal-acetate buffer, pH 8.5, $\mu = 0.1$, employing a current of 4 V. per cm. The Whatman No. 1 paper strips were stained with amidoschwarz 10B (Bayer, Leverkusen). The stained strips were soaked in bromonaphthalene-paraffin and then scanned in a special colorimeter (Zeiss, Oberkochen); the curves were evaluated planimetrically.

Brief summary of the clinical condition (1–5)

The female analbuminemic subject, a peasant's daughter who was born in 1922, came into the clinic with edema in her ankles. She was sent by her physician for investigation of a high blood sedimentation rate (between 42/68 and 95/104 mm. Westergren) and a hypotension of 90/55 mm. Hg. The edema appeared regularly before and during menstruation. Other clinical and biochemical data were: blood calcium, 8.2 to 8.5 mg. per cent; Takata-Ara, thymol turbidity, Weltmann's $CaCl_2$ coagulation, and $CuSO_4$ reactions strongly positive. Volhard's diuresis test (water shock) showed an excessive diuresis, but normal concentration values. The cholesterol, total lipoid, hexosamine, protein-bound hexose, and choline esterase values in the serum were elevated. Both colloid osmotic and capillary pressures were low. Normal serum values were found for potassium, phosphorus, urea, uric acid, nonprotein nitrogen, creatinine, bilirubin, chlorides, alkaline phosphatase and prothrombin. Normal values were also found in some liver function tests, *i.e.*, galactose, "Testacid®," and bromosulfalein. Some renal clearance tests showed pathological changes, *e.g.*, *p*-aminohippuric acid and inulin.

Total serum protein was 4.6 Gm. per 100 ml.; the relative composition of the globulins averaged as follows:

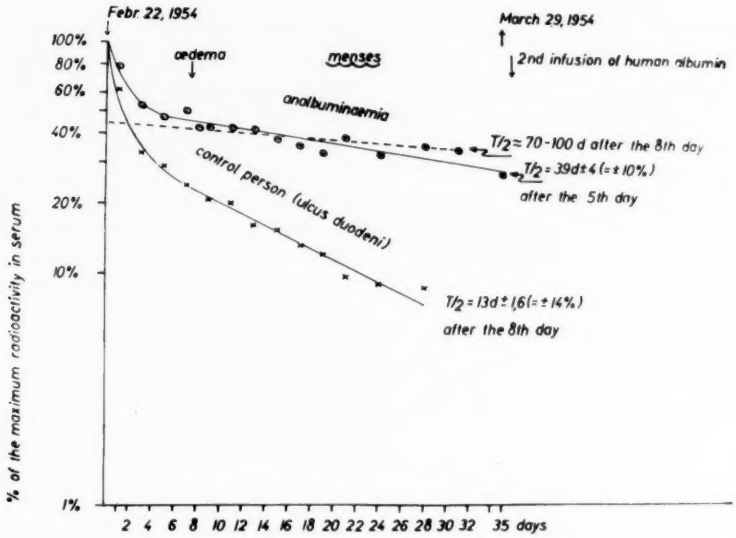


FIG. 2. COMPARISON OF THE DISAPPEARANCE FROM BLOOD STREAM OF A LABORATORY PREPARATION OF I^{131}-ALBUMIN IN A CASE OF ANALBUMINEMIA (○—○) AND IN A CONTROL SUBJECT (×—×)

Ordinate: serum radioactivity in per cent of the maximum serum radioactivity 15 minutes after the injection. Abscissa: time elapsed after the injection.

α_1, 7.4 per cent; α_2, 19.7 per cent; β, 32.1 per cent; γ, 40.8 per cent (Figure 1). These values varied within the limits of methodological error. No albumin could be detected by use of both free and paper electrophoresis, ultracentrifuge, or immunological experiments within the limits of the specificity of these methods, *i.e.*, less than 1 per cent of the total serum protein.

Evans blue disappeared at an almost normal rate during the first two hours, but afterwards more rapidly. Congo red disappeared at the high rate of 70 per cent from the blood stream within one hour as compared with 30 per cent in normal subjects (9). Such extremely high rates are usually encountered in amyloidosis only. In this instance, however, the rapid disappearance is caused by the deficiency of albumin to which congo red is normally bound. This is a striking example of how the presence of albumin vehicles (32) normally affects the disappearance rates of some substances which are circulating in the blood stream.

The family tree bears several intermarriages (3). We conclude from it that complete deficiency of albumin is a highly recessive hereditary trait.

The exact data are contained in earlier papers (1–4, 10–13), or will be published in due course (5).

RESULTS

I) Preliminary experiments

The analbuminemic subject was injected intravenously with 50 Gm. of noncrystallized human serum albumin for the first time on June 10, 1953, when she was 31 years old and weighed 73.3 Kg. This first injection was done within half an hour. Two hundred and twelve days later, on Feb. 22, 1954, 30 mg. of I^{131}-tagged albumin containing 325 μc. of I^{131} was injected. On this day, her blood still contained about 0.15 Gm. per cent albumin from the first injection, and hemoglobin was 80 per cent (or 12.8 Gm. per 100 ml.). The plasma volume was about 3.6 L. as determined with I^{131} albumin. Total protein nitrogen was 5.1 Gm. per 100 ml.

During the first five to eight days after the injection of I^{131}-albumin, the radioactivity per milliliter of serum decreased more rapidly than it did subsequently (Figure 2). This initial high rate of decline is in conformity with the literature and has been ascribed mainly to establishment of an equilibrium between intra- and extravascular spaces (14–16). After the first five to eight days, the slope of the curve changed to an approximately straight line. The points of this line can be connected in such a way as to yield half-life figures of either 35 to 43 days (1–3) or up to as much as 70 to 100 days. No correction was made for a loss of

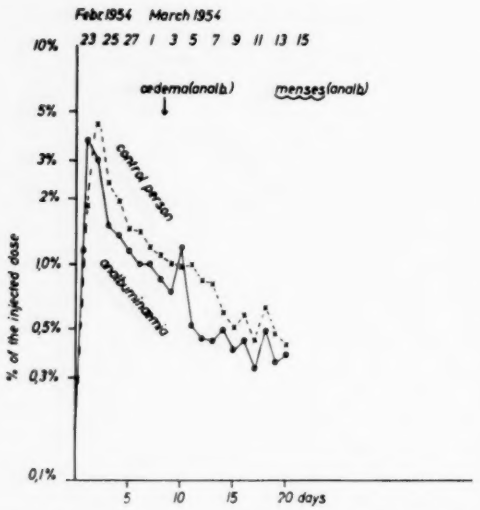


FIG. 3. DAILY URINARY EXCRETION RATES OF RADIOACTIVITY AFTER THE FIRST INJECTION OF I^{131}-ALBUMIN

The TCA-precipitable radioactivities were less than 1 per cent of the total radioactivities excreted in both cases. Ordinate: per cent of the injected total radioactivity. Abscissa: time. Analbuminemic subject (○—○); control (×- - -×).

radioactivity of about 5 per cent due to withdrawal of blood samples. The half-life of the same preparation of I^{131}-albumin in a control subject averaged 13 days.

During the first 20 days after the injection of I^{131}-albumin, the analbuminemic subject excreted only about 20 per cent of the injected radioactivity in the urine (Figure 3). For want of a sufficiently sensitive Geiger counter, we were unable to measure exactly the radioactivity of the thyroid. But from some isolated measurements and from data given in the literature (17) we may assume that the uptake of I^{131} by the thyroid is negligibly low (not exceeding 5 to 10 per cent).

After the injection of commercial human serum albumin nine months earlier, the half-life of this nonradioactive albumin was also about 70 (60 to 80) days, as determined by paper electrophoresis and immunological methods (Figure 4). Thus, it appeared that the half-life of albumin in this subject is much longer than in normals, and the values given by the two different methods are similar.

II) Second series

The determination of the half-life of I^{131}-albumin was repeated in the subject between May 10 and June 30, 1957. This time the subject's serum contained almost indetectable traces of albumin from former infusions; total serum protein was 5.0 Gm. per 100 ml. ($CuSO_4$ method); hemoglobin, 94 per cent (or 15.0 Gm. per 100 ml.). Hematocrit values averaged 42 per cent. Only small amounts of blood (6 to 10 ml. samples) were taken in comparatively long time intervals. Instead of a laboratory-made preparation of I^{131} albumin, we injected 6 mg. of the commercial I^{131}-labeled albumin, RISA® (Abbott), containing 60 μc. of I^{131}. This time, plasma volume was 3.0 L. We were now able to follow the disappearance of radioactivity for 51 days by using a well-type scintillation counter (Figure 5). The paper electrophoresis strips were now evaluated, taking due care to exclude nonmigrating paper-bound and denatured protein (18, 19) when present (in traces).

On the second and third days after the injection of RISA®, 205 Gm. of nonradioactive human serum albumin[2] was infused into the subject, 175 Gm. within 11 hours. As a result of this main infusion of 175 Gm. of albumin dissolved in 1.2 L. of saline solution and 2.3 L. of 5 per cent glucose solution, the plasma volume increased during the day, causing a drop in radioactivity from 45 to 32 per cent of the value found 10 minutes after the injection (Figure 6). This fall in plasma radioactivity is equivalent to an increase of plasma volume from approximately 3.0 to about 4.2 L. During both infusions, and during the night following the main infusion, the subject excreted a total of 5.9 L. of urine within 48 hours. After the main infusion the plasma radioactivity rose overnight to 37 per cent, this value again lying close to the slope of the curve of the initial disappearance, where one would have expected it to lie had no additional albumin been infused (Figure 6). The body weight remained unchanged at 69.5 Kg. These findings mean that the main part of the original plasma volume had been restored overnight.

30 Gm. of albumin had been infused within four hours on the day before the main infusion

[2] Lots No. 1118 n and 386 (Behringwerke). These lots had been manufactured on Nov. 1, 1956, and Dec. 12, 1952, respectively, and had not been crystallized.

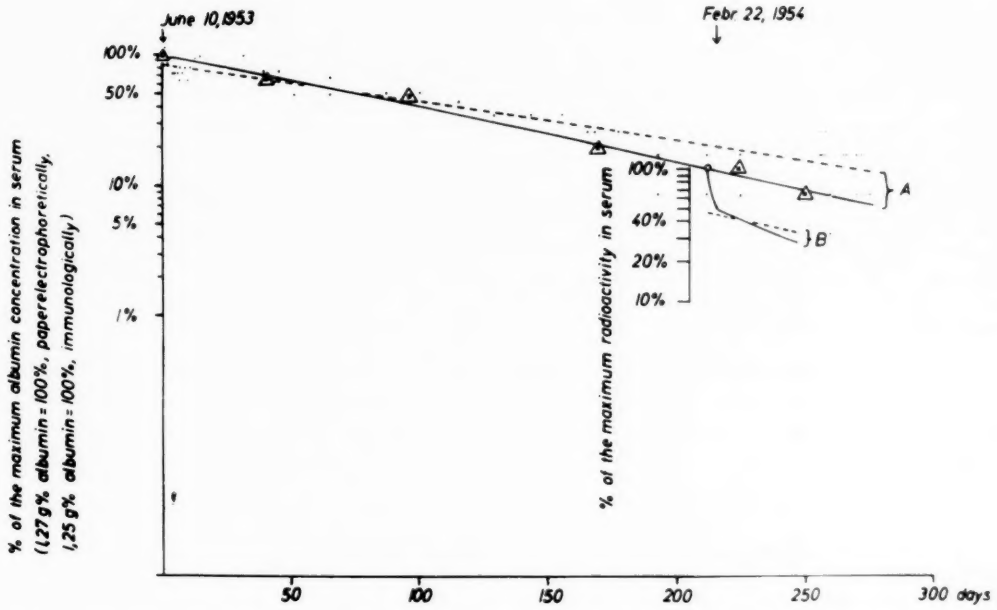


FIG. 4. HALF-LIFE CURVES OF UNLABELED HUMAN SERUM ALBUMIN (STRAIGHT LINES, A) AFTER THE FIRST INJECTION OF I^{131} ALBUMIN INTO THE ANALBUMINEMIC SUBJECT

·- - - - · indicates paper electrophoretic values; △——△ immunological values.

The auxiliary ordinate on the right is for the I^{131}-albumin (B) and is drawn in the same scale as the main ordinate. The B curves are identical with the upper two curves in Figure 2, but with the scale of the abscissa reduced five times. For the original points of Curve B and the manner in which the two seemingly different lines can be drawn, see Figure 2.

Half life values of (A) nonradioactive albumin are 70 ± 10 days (solid line), or 90 to 110 days (broken line); (B) I^{131}-albumin, 39 ± 4 days (solid line), or 70 to 100 days (broken line).

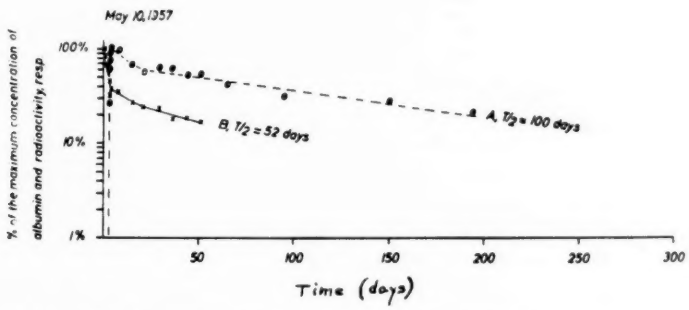


FIG. 5. HALF-LIFE CURVE OF HUMAN SERUM ALBUMIN (○—○) AFTER THE INFUSION OF 205 GM. (30 + 175 GM.) OF NONLABELED ALBUMIN

Two and ninety-five one-hundredths Gm. per cent albumin in the serum equals 100 per cent in Curve A (○—○). The half-life curve, B (×—×), after the injection of 6 mg. of I^{131}-albumin (RISA®) containing 60 μc. of I^{131} is also plotted. Note the sudden drop and rise of radioactivity following the infusion of serum albumin (*cf.* Figure 6).

This was the fifth of a series of infusions in the course of four years.

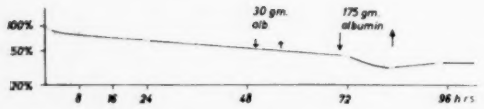


FIG. 6. DISAPPEARANCE FROM BLOOD STREAM OF I^{131} ALBUMIN (RISA®) DURING THE FIRST FOUR DAYS AFTER INJECTION

This curve is the same as Curve B in Figure 5, except that the abscissa is drawn on a much wider scale. The arrows indicate the beginning and end of the additional infusions of 30 and 175 Gm., respectively, of non-radioactive albumin. Note the relatively sudden changes of plasma volume within a few hours.

(Figure 6). Compared with the 175 Gm. which was infused during the main infusion, the effect of this 30 Gm. of albumin on the plasma volume may be neglected.

Whereas the curve of disappearance of infused albumin in Figure 5 shows only a slight initial fall in the concentration of albumin, it is necessary to emphasize that this may arise from the artificial nature of the 100 per cent origin. Because this large amount of albumin was infused slowly over 11 hours and was associated with transcapillary migrations of water and of protein of an unpredictable nature, the starting albumin level of 2.95 Gm. per 100 ml. has no absolute significance. The absence of a sharp initial fall in plasma albumin concentration in this curve and in the ones in Figures 4 and 8 should not be taken to mean that this analbuminemic subject has an abnormally small amount of extravascular fluid. Indeed, when I^{131}-albumin was injected instantaneously in tracer amounts, the rapid initial fall in plasma radioactivity suggested that the subject had a substantial extravascular fluid space available to albumin.

III) Third series

In order to decide definitely upon the questions arising from the differences which we had found in the first two experiments, we arranged a third experiment in co-operation with Drs. A. S. McFarlane, C. M. E. Matthews, T. Freeman (National Institute for Medical Research, London) and Prof. H. E. Schultze (Behringwerke, Marburg). For this purpose, the Behringwerke prepared a special lot of highly purified human albumin to which no preservative had been added. A sample of this albumin was jet-iodinated by Dr. McFarlane on July 18, 1958.

On July 20, 1958, the analbuminemic subject

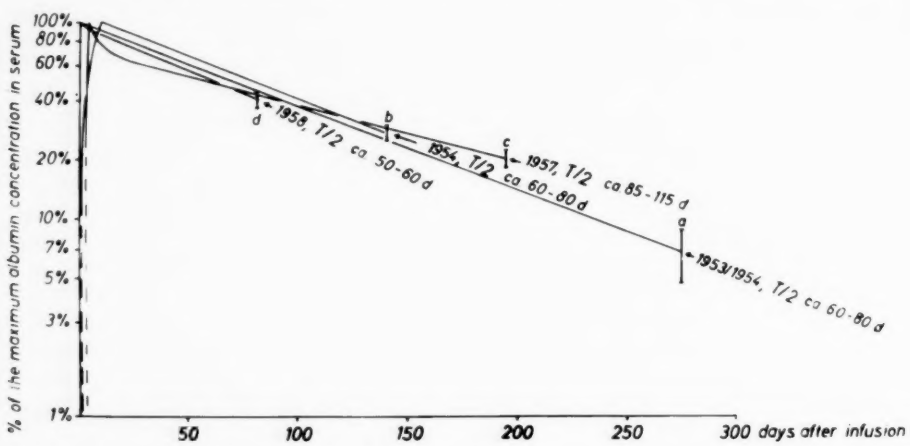


FIG. 7. SUMMARY OF DIFFERENT HALF-LIFE CURVES OBTAINED FROM EXPERIMENTS WITH UNLABELED HUMAN ALBUMINS IN THE ANALBUMINEMIC SUBJECT BETWEEN 1953 AND 1958

The perpendicular bars indicate the approximate mean error of the paper electrophoretic determinations. *a*, *b* and *c*: uncrystallized human albumins to which mercury-containing preservatives were added; *d*: crystallized human albumin without preservative (first part of the preliminary observation period: only 89 days).

It is evident that these arbitrary half-life values are overlapping and, therefore, do not differ significantly from one another.

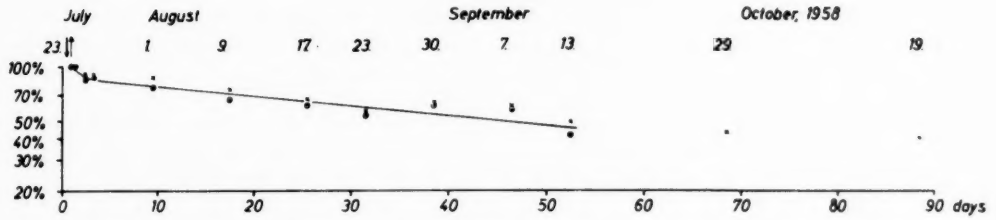


FIG. 8. HALF-LIFE CURVE OF BOTH NONLABELED ALBUMIN (×—×) AND JET-IODINATED I^{131}-ALBUMIN (⊙—⊙) AFTER THE INFUSION OF A MIXTURE OF BOTH ALBUMIN PREPARATIONS WITHIN NINE HOURS

Ordinate: per cent of the maximum concentration of human serum albumin (2.63 Gm. per 100 ml.) and radioactivity, respectively. Abscissa: time, in days. Arrows indicate the beginning and end of the infusion of 145 Gm. of human serum albumin (Behringwerke) containing 68.5 μc. of jet-iodinated I^{131}-albumin.

The half-life (about 50 to 60 days) and initial disappearance of both preparations are practically identical.

came again to the hospital with declining edema which had almost disappeared from her ankles on the day prior to the infusion of albumin. This time, no albumin was preinfused. Total serum protein was 4.8 Gm. per cent (biuret method); hemoglobin, 90 per cent (14.4 Gm. per 100 ml.); and hematocrit, 40 per cent.

On July 23, 1958, 68.5 μc. of I^{131}-albumin (about 50 mg.) was added to 148 Gm. of Behring-albumin (Lot No. 1321 n) in 740 ml. of a solution containing 0.5 per cent NaCl and 0.4 per cent $NaHCO_3$ in water. Two hundred fifty ml. of this mixture was made up to 1,000 ml. with 0.9 per cent saline solution; the rest was diluted with 5 per cent glucose solution to a volume of 2,000 ml. This total of 3,000 ml. of 5 per cent albumin solution was infused into the fasting subject on the same day between 11:30 a.m. and 8:20 p.m. At 6 p.m. she received a light supper which she vomited two minutes after the infusion was over. Otherwise, the patient was doing well.

During the following observation period of 52 days, a total of about 300 ml. of blood was taken. In the serum and plasma samples, radioactivity was measured in the same way as in the 1957 experiment. The albumin content was determined immunologically and by paper electrophoresis.

The half-lives of both labeled and unlabeled human serum albumin were essentially identical, a result which Dr. McFarlane and his co-workers had anticipated from previous experience with labeled albumins and from the fact that the labeled molecules were mixed in this experiment with the unlabeled ones prior to the infusion. The specific radioactivities of the albumin did not change significantly during the observation period of seven and one-half weeks (20). As on previous occasions, only an extremely small apparent disappearance took place during the first five days, again probably because the albumin was infused rather than injected with a corresponding uncertainty in the significance of the origin.

The results (Figure 8) support the following conclusions: *1*) The subject's protein metabolism was unable to discriminate between unlabeled albumin and the same albumin labeled by Dr. McFarlane's (21) procedure. Therefore, half-life values determined by use of this labeled albumin have some metabolic significance.

2) The I^{131}-albumin used in February, 1954, was probably also undenatured because it behaved in a normal fashion in the control subject; in addition, there was only a relatively slight initial fall in plasma radioactivity during the first six to eight days following the injection.

3) The I^{131}-albumin used in May, 1957, was probably abnormal (20, 22) since there was an unusually large initial fall in plasma radioactivity and the half-life was significantly shorter than that of the unlabeled protein.

DISCUSSION

Half-life values of 25.6 to 65 days for S^{35}- or C^{14}-labeled human serum albumins were found by Niklas and Maurer (23), Niklas and Poliwoda (24), Armstrong and co-workers (25, 26), Masouredis and Beeckmans (27), and Margen and Tarver (28). Margen and Tarver (28) have observed that the metabolic half-life of the I^{131}-label

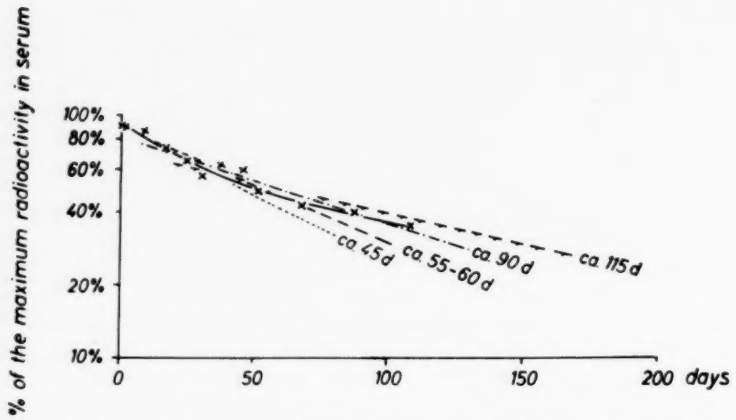


FIG. 9. DIFFERENT POSSIBILITIES OF EVALUATING THE 1958 PAPER ELECTROPHORETIC HALF-LIFE CURVE WITHIN A PRELIMINARY OBSERVATION PERIOD OF 109 DAYS

is considerably lower than that of C^{14} or S^{35} labels in the same animals or subjects. The half-life curves in our second experiment obtained with I^{131}-labeled and unlabeled human albumin could conform in principle with some of these authors' findings. On the other hand, the results of our first and third experiments do not.

More recently McFarlane (14) and co-workers have found no difference in half-lives when using mixtures of I^{131}- and C^{14}-labeled serum proteins in animals. In view of the lack of agreement in our first two experiments on the analbuminemic subject, we still had some doubts as to the reliability of breakdown rates obtained by the use of iodinated proteins in general (29). Therefore, we undertook a crucial third experiment in co-operation with Dr. McFarlane and his co-workers. In our opinion, it has now been shown that, provided sufficient care is taken in the preparation of the labeled human albumin, the use of the I^{131}-label is justified for metabolic studies. As we have shown in this paper, both the initial disappearance rate and half-life of the labeled albumin are identical with the values obtained by use of the non-labeled protein.

Miller and Bale (30) have shown conclusively that serum albumin is synthesized and metabolized in the liver. As is evident from our experiments, both synthesis and breakdown of albumin seem to be linked together in some way.

Since the catabolism of albumin is only slowed, but not blocked, in this case of analbuminemia, there must exist several ways by which albumin can be metabolized by the liver in general: *a*) a process highly specific for albumin, which apparently fails to work in this subject; *b*) a non-specific catabolism of albumin which has retained its efficiency; *c*) a process associated with the integrity of albumin molecules. As we have shown in our second experiment, denatured albumin molecules disappear more rapidly from the blood stream than intact molecules; *d*) since the analbuminemic subject does not exhibit symptoms of myxedema or other severe endocrinological disorders, the influence of the thyroid (31, 20) and other hormones on the catabolism of proteins may be disregarded in the interpretation of the half-life values in this case.

In any event, the half-life of albumin is definitely much longer in this case of analbuminemia than in normals. However, we have found a considerable latitude in drawing the half-life curves of infused albumin (*cf.* Figures 2, 4 and 9); therefore, we are unable to determine the half-life in this subject more exactly than within a range of between 40 and 110 days (Figures 2, 7 and 9), apparently depending only on the time period during which the I^{131}-albumin content of plasma can be measured exactly. Similarly, deductions regarding extravascular space cannot be made with any accuracy if substantial amounts of albumin are infused over a significant time interval. This arises mainly from unpredictable shifts of fluid and al-

bumin which obscure the true origin ("100 per cent") in the graphs.

SUMMARY

1) The half-life values of I^{131}-albumin and nonradioactive human serum albumin were compared in a case of analbuminemia.

2) In one experiment using a commercially available I^{131}-albumin, the half-life values and initial disappearance rates differed considerably.

3) In two other experiments, one of them using an albumin which had been labeled for us by Dr. A. S. McFarlane, both the half-lives and initial disappearance rates of labeled and unlabeled albumin were identical.

4) Some implications of these findings are discussed.

ACKNOWLEDGMENTS

The authors are indebted to Drs. A. S. McFarlane, C. M. E. Matthews and T. Freeman for generously providing us with iodinated I^{131}-albumin and for a critical review of the manuscript. We also wish to thank Mrs. L. Tappe for drawing the diagrams.

REFERENCES

1. Bennhold, H., Peters, H., and Roth, E. Ueber einen Fall von kompletter Analbuminaemie ohne wesentliche klinische Krankheitszeichen. Verh. dtsch. Ges. inn. Med. 1954, **60**, 630.
2. Bennhold, H. Die Bedeutung der Bluteiweisskörper fuer den Wasserhaushalt. Regensburg Jb. ärztl. Fortbild. 1954/55, No. **IV**, 72.
3. Bennhold, H. Kongenitale Defektdysproteinämien. Verh. dtsch. Ges. inn. Med. 1956, **62**, 657.
4. Ott, H. Das Blutserum bei Analbuminämie. Weitere Untersuchungen über die Serumfraktionen, das Farbstoffbindungsvermögen und den kolloidosmotischen Druck. Z. ges. exp. Med. 1957, **128**, 340.
5. Bennhold, H., and Scheurlen, G. In preparation.
6. Banks, H. H., Seligman, A. M., and Fine, J. The effect of hyaluronidase on the absorption of parenterally administered radioactive plasma proteins in the dog. J. clin. Invest. 1949, **28**, 548.
7. Heidelberger, M., and Kendall, F. E. The precipitin reaction between type III pneumococcus polysaccharide and homologous antibody. III. A quantitative study and a theory of the reaction mechanism. J. exp. Med. 1935, **61**, 563.
8. Peters, J. P., and Van Slyke, D. D. Quantitative Clinical Chemistry. Vol. II, Methods. Baltimore, Williams & Wilkins, 1932. [Cit. by Lempp, R. Über die Fehlerbreite der van Slyke' schen Kupfersulfatmethode zur quantitativen Serumeiweissbestimmung im Vergleich mit anderen Verfahren. Dissertation, Tübingen, 1950.]
9. Bennhold, H. Ueber die Ausscheidung intravenoes einverleibten Kongorotes bei den verschiedensten Erkrankungen, insbesondere bei Amyloidosis. Dtsch. Arch. klin. Med. 1923, **143**, 32.
10. Scheurlen, P. G. Untersuchungen ueber die Regulation der Serumeiweisskoerper. Medizinische 1958, No. 33/34, 1246.
11. Bennhold, H., Ott, H., and Scheurlen, P. G. Beitraege zur Frage der genbedingten Bluteiweißstörungen. Verh. dtsch. Ges. inn. Med. 1958, **64**, 279.
12. Lohss, F., and Kallee, E. Spurennachweis von Albumin durch Analyse von Antigen-Antikoerperpraecipitaten. Clin. chim. Acta 1959, **4**, 127.
13. Freeman, T., Matthews, C. M. E., McFarlane, A. S., Bennhold, H., and Kallee, E. ^{131}I-albumin in an analbuminemic subject. Nature (Lond.) 1959. In press.
14. McFarlane, A. S. Use of labeled plasma proteins in the study of nutritional problems (a review). Progr. Biophysics 1957, **7**, 115–163.
15. Matthews, C. M. E. The theory of tracer experiments with ^{131}I-labelled plasma proteins. Phys. in Med. Biol. 1957, **2**, 36.
16. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J. Tracer experiments with I^{131} labeled human serum albumin: Distribution and degradation studies. J. clin. Invest. 1953, **32**, 746.
17. Storaasli, J. P., Krieger, H., Friedell, H. L., and Holden, W. D. The use of radioactive iodinated plasma protein in the study of blood volume. Surg. Gynec. Obstet. 1950, **91**, 458.
18. Bennhold, H., Kallee, E., and Roth, E. Autoradiographisches Verfahren zur qualitativen papierelektrophoretischen Auftrennung kleiner und kleinster Eiweissmengen. Z. Naturforschg. 1952, **7b**, 661.
19. Scheurlen, P. G. Beitrag zur Auswertung der Papierelektrophoresestreifen mit Integrationsgeräten. Klin. Wschr. 1957, **35**, 485.
20. Iber, F. L., Nassau, K., Plough, I. C., Berger, F. M., Meroney, W. H., and Fremont-Smith, K. The use of radioiodinated albumin in metabolic studies. The effects of the level of dietary protein and L-triiodothyronine on the catabolism of radioiodinated human serum albumin. J. clin. Invest. 1958, **37**, 1442.
21. McFarlane, A. S. Labelling of plasma proteins with radioactive iodine. Biochem. J. 1956, **62**, 135.
22. Steinfeld, J. L., Greene, F. E., Tabern, D. L., Paton, R. R., and Flick, A. L. Degradation of iodinated human serum albumin prepared by various procedures. J. Lab. clin. Med. 1958, **51**, 756.
23. Niklas, A., and Maurer, W. Messung der Neubildungs- und Abbaugeschwindigkeit einzelner Serumeiweissfraktionen nach Gabe von S^{35}-Methionin. Verh. dtsch. Ges. inn. Med. 1953, **59**, 319.
24. Niklas, A., and Poliwoda, H. Zur Frage der biologischen Halbwertszeit menschlicher Albumine und Globuline. Biochem. Z. 1954, **326**, 97.

25. Armstrong, S. H., Jr., McLeod, K., Wolter, G., and Kukral, J. The persistence in the blood of the radioactive label of albumins, gamma globulins, globulins of intermediate mobility studied with S^{35} and paper electrophoresis: Methods and preliminary results. J. Lab. clin. Med. 1954, **43**, 918.

26. Armstrong, S. H., Jr., Bronsky, D., and Hershman, J. The persistence in the blood of the radioactive label of albumins, gamma globulins and globulins of intermediate mobility. III. Comparison of die-away plots following oral and intravenous administration of the S^{35} label to the same subjects. J. Lab. clin. Med. 1955, **46**, 857.

27. Masouredis, S. P., and Beeckmans, M. L. Comparative behavior of I^{131} and C^{14} labelled albumin in plasma of man. Proc. Soc. exp. Biol. (N. Y.) 1955, **89**, 398.

28. Margen, S., and Tarver, H. The deiodination of proteins labeled with I^{131}. Ann. N. Y. Acad. Sci. 1957, **70**, 49.

29. Freeman, T., and Matthews, C. M. E. Analysis of the behaviour of I^{131}-albumin in nephrotic patients (with discussion remarks by Kallee, E.) *in* Radioaktive Isotope in Klinik und Forschung. Vienna, Verlag Urban & Schwarzenberg, 1958, vol. III, 306.

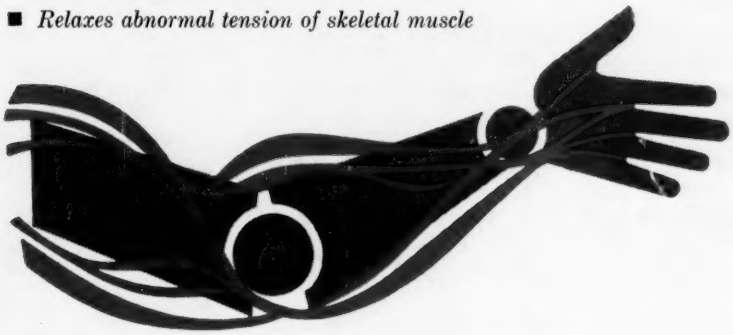
30. Miller, L. L., and Bale, W. F. Synthesis of all plasma protein fractions except gamma globulins by the liver. J. exp. Med. 1954, **99**, 125.

31. D'Addabbo, A., and Kallee, E. ^{131}J-Serumproteine und Schilddrüse. Z. Naturforschg. 1959, **14b**, 240.

32. Bennhold, H. Ueber die Vehikelfunktion der Serumeiweisskoerper. Ergebn. inn. Med. Kinderheilk. 1932, **42**, 273.

now...a new way to relieve pain and stiffness in muscles and joints

- *Exhibits unusual analgesic properties, different from those of any other drug*
- *Specific and superior for relief of* SOMA*tic pain*
- *Modifies central perception of pain without abolishing natural defense reflexes*
- *Relaxes abnormal tension of skeletal muscle*



SOMA T.M.

N-isopropyl-2-methyl-2-propyl-1, 3-propanediol dicarbamate

In back pain, bursitis, sprains, strains, and bruises, whiplash and other traumatic injuries, inflammatory and degenerative muscle and joint complaints.

RAPID ACTING. Pain-relieving and relaxant effects start within 30 minutes and last for at least 6 hours.

NOTABLY SAFE. Toxicity is extremely low. No effects on liver, endocrine system, blood pressure, blood picture or urine have been reported. Some patients may become sleepy on higher than recommended dosage.

EASY TO USE. Usual adult dose is one 350 mg. tablet 3 times daily and at bedtime.

SUPPLIED: Bottles of 50 white sugar-coated 350 mg. tablets.

Literature and samples on request.

WALLACE LABORATORIES, NEW BRUNSWICK, N. J.

REVUE FRANÇAISE D'ÉTUDES CLINIQUES ET BIOLOGIQUES

APRIL, 1959

Vol. IV, No. 4

Editorial

Fonction erythropoïetique du rein. J. P. NAETS.

Original Articles

Comparaison des résultats fournis par trois méthodes de dosage du cholesterol serique (Schoenheimer-Sperry, Grigaut, Delsal). D. BARGETON, M.-E. TRICAUD-REDEL, P. GROS.

Etude de l'action des 4 dérivés du cholestérol sur des cellules vivantes cultivées "In vitro." H. LETTRE, O. FLANDRE.

Corticosteroïdes sanguins chez le chien, au cours de l'ischémie musculaire traumatique—Etude comparative de la corticoïdémie sur des prélèvements réalisés dans les veines cave, périphérique, voire surrénalienne. A. DE SCOVILLE, H. VAN CAUWENBERGE, M. PALEMVLIERS.

Les transaminases du sérum en pathologie hepato-biliaire. P. BOIVIN, R. BESSON, L. HARTMANN, R. FAUVERT.

Original Notes

Afibrinémies acquises. J. CAEN, M.-J. LARRIEU, J. BERNARD.

Para-gamma globulinémie révélées par l'électrophorèse à travers gel d'amidon. A. MARNAY, J. MORETTI, M.-F. JAYLE.

Mise en évidence de l'antigène KELL dans les plaquettes sanguines humaines. J. DUCOS, J. BROUSSY, J. RUFFIE.

Essai d'absorption de certains anticorps par des hématies hémolysées. II. Utilisation de l'acide acétique pour obtenir des stromas globulaires dépourvus d'antigènes de groupe actif. J. DUCOS, J. RUFFIE.

Etude radio-clinique d'une gynecomastie oestrogénique. R. AMALRIC, S. GELLER.

General Review

La protéolyse sanguine. Son mécanisme, son exploration par les méthodes actuelles et sa physiopathologie. H. YATZIDIS.

Technical Note

Technique de mesure des effets de substances pharmacodynamiques sur le métabolisme basal des petits animaux. MME O. THIBAULT.

Subscription rate, $19.00

Special rate for interns and residents, $13.00

EDITIONS MEDICALES FLAMMARION, 22, rue de Vaugirard, PARIS 6ème. France.

NOT 800 SUPPLIERS—

JUST ONE

That's what Harshaw Scientific offers the laboratories—
ONE SOURCE for practically their entire requirements

This represents *many thousands* of items —no matter how large nor how inexpensive—from electron microscopes to corks—obtained from over 800 manufacturers in all parts of the country.

And these items are *available from stock* in our *six* branch warehouses.

Actually we are the laboratory's SUPER MARKET. The saving in reducing the number of orders placed is tremendous. A recent survey showed that a number of routine customers' orders included as many as 12 to 30 different manufacturers' items. The increased cost of issuing 12 to 30 orders instead of 1 is obviously considerable. Add to that transportation charges from out of the way locations, plus delay in deliveries and the increase in cost cannot be estimated.

We take our job very seriously. Each item is carefully evaluated before being added to our line. Most items are included in our current catalog. As new items become available we attempt to bring them to your attention through space ads and our house organs.

Our branches and sales offices are staffed with personnel trained to be helpful. They have a genuine interest in your problems. In addition we believe you will enjoy dealing with them. We think they're fine people.

If you are not taking full advantage of our facilities, we would welcome an opportunity to serve you.

No matter what you need—furniture, glassware, apparatus, chemicals or equipment, you can get it from HARSHAW.

HARSHAW

Branches

CLEVELAND 6, OHIO
1945 East 97th Street
Tel. VUlcan 3-2424

CINCINNATI 13, OHIO
6265 Wiehe Rd.
Tel. REdwood 1-9100

DETROIT 28, MICHIGAN
9240 Hubbell Avenue
Tel. VErmont 6-6300

HOUSTON 11, TEXAS
6622 Supply Row
Tel. WAlnut 3-1627

LOS ANGELES 22, CALIF.
3237 So. Garfield Ave.
Tel. RAymond 3-3161

PHILADELPHIA 48, PA.
Jackson & Swanson Sts.
Tel. HOward 2-4700

Sales Offices

AMARILLO, TEXAS
3409 S. Jackson Street

ATLANTA 5, GEORGIA
3130 Maple Drive, N.E.
Tel. CEdar 3-3560

BATON ROUGE 6, LA.
3160 Florida Street
Doherty Bldg., Room 103
Tel. Dickens 3-1933

BUFFALO 2, NEW YORK
260 Delaware Avenue
Tel. GArfield 9-2000

HASTINGS-ON-HUDSON 6
NEW YORK
Tel. HAstings 5-8250

OAKLAND 11, CALIFORNIA
3826 Piedmont Avenue
Tel. OLympic 5-6511

PITTSBURGH 22, PA.
504 Bessemer Building
6th St. & Fort Duquesne Blvd.
Tel. ATlantic 1-7930

HARSHAW SCIENTIFIC
Division of
The Harshaw Chemical Company
Cleveland 6, Ohio

"We Count More Samples . . .
Count For Longer Periods . . .
And Still Have More Time For Research"

Tri-Carb® Liquid Scintillation Spectrometer

For counting Tritium, Carbon-14 and other beta- and alpha-emitting isotopes.

Auto-Gamma® Spectrometer

for counting gamma-emitting isotopes.

Simply Load Up To 100 Samples And Read The Printed Data

This is a typical quote from research personnel in laboratories where Packard Tri-Carb Spectrometers and/or Auto-Gamma Spectrometers are used.

These instruments are completely automatic. They handle up to 100 samples and record all data (sample number, time *and scaler counts*) accurately and permanently on paper tape. Operation can be maintained on a 24-hour basis. No staff time is required for counting. Consequently, laboratory personnel have more time for other important research work.

If you are counting radioactive samples, learn how Packard can improve your experimental data and still save you time. Contact Packard Instrument Co., Inc. Request latest bulletin on the Tri-Carb Liquid Scintillation Spectrometer and/or the Auto-Gamma Spectrometer.

Packard *Instrument Company, Inc.*

DEPT. J • P.O. BOX 428 • LA GRANGE, ILLINOIS

San Francisco • Chicago • New York

LANCASTER PRESS, INC., LANCASTER, PA.